Phytochrome Regulation of Root Development in *Arabidopsis thaliana*

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ABSTRACT

Analysis of phytochrome null mutants has revealed roles for these photoreceptors throughout plant development, synchronising developmental events to daily and seasonal environmental changes. The roles of the phytochromes in shoot photomorphogenesis is relatively well characterised, but little is known about their influence on root development. Over the course of my PhD I have shown that phytochromes are in fact important regulators of root phenotype. My results demonstrate that application of low R:FR ratio light, which reduces the active phytochrome pool (Pfr), changes the distribution of expression of the synthetic auxin reporter gene *DR5::*GUS, which in turn correlates with a reduction in lateral root emergence. Thus, I have identified a long distance signalling role for shoot phytochromes, acting collectively to control the emergence of lateral roots, at least partly by manipulating the early shoot-root auxin pulse. I have shown that phytochromes are present and light regulated in roots, and propose that root phytochromes are able to act locally to regulate root hair elongation. My analysis of the interaction between *phyB* and *shy2-2* implicates these genes in the regulation of microtubule stability, and consequently of cytoskeleton organisation. I have also provided evidence that the Pr form of phytochrome, previously thought to be physiologically inactive, does in fact have important roles in the regulation of root development. I have taken a novel approach to understanding phytochrome signalling, and have opened many new lines of enquiry into an exciting new area of photobiology.

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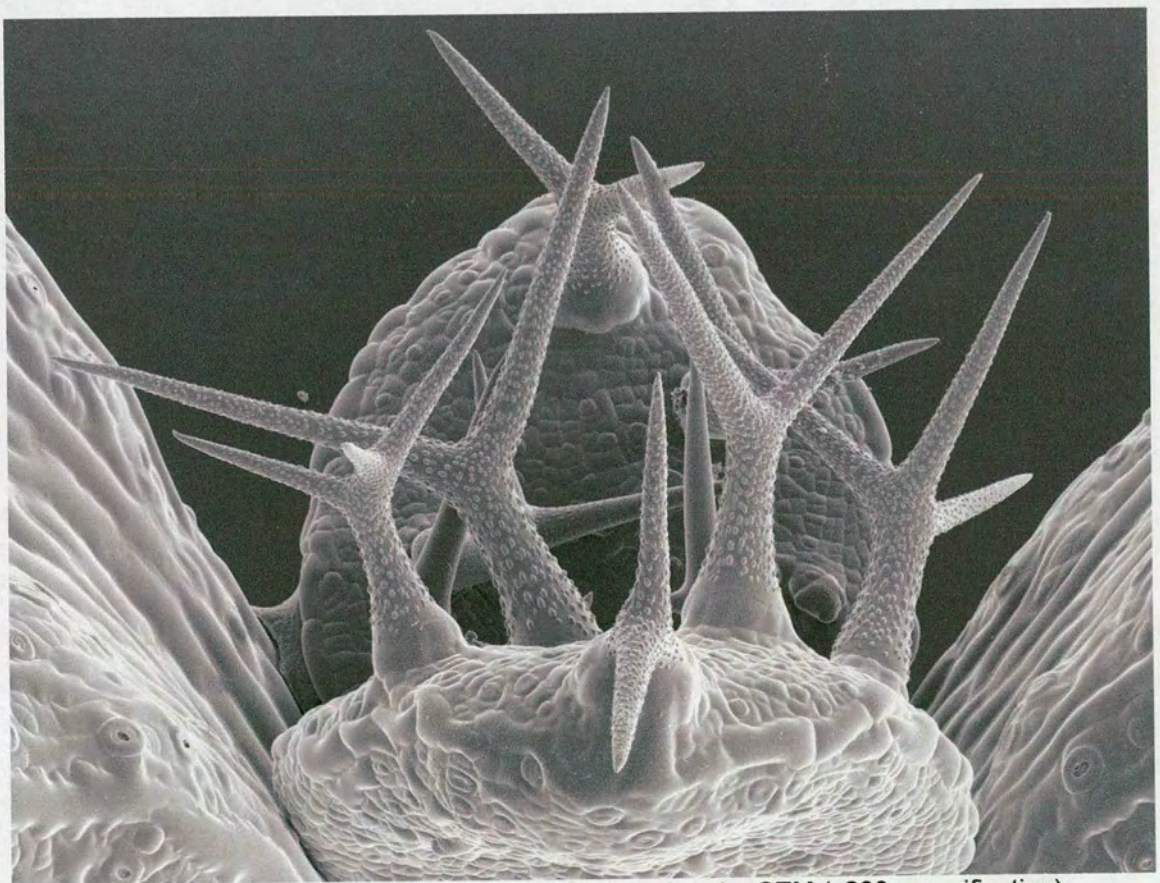
Introduction



Trichomes of a 7 day old wild type seedlings viewed under SEM (x200 magnification)

CHAPTER 1

INTRODUCTION

Plants have evolved complex signalling systems to connect sensory inputs with developmental pathways. Such networks ensure development is synchronised to local and seasonal changes in the environment, and that it proceeds in an appropriate and timely fashion. As photoautotrophs, accurate perception and suitable response to light is of utmost importance. To this end, plants have evolved a suite of photoreceptors and a complex signalling network that allows a coordinated response to changes in the light environment. In Arabidopsis there are at least 3 major groups of photoreceptors, comprising the red and far-red light absorbing phytochromes (Whitelam *et al.*, 1998), the UV-A/blue light absorbing cryptochromes (cry1–3), and phototropins (phot1 and 2) (Briggs and Christie, 2002; Lin, 2002). There is also evidence for a presently uncharacterised photoreceptor, acting in response to UV-B light (Brown *et al.*, 2005).

Phytochromes Act Together To Regulate Responses to Red and Far Red Light

In Arabidopsis, the phytochromes form a small gene family (phyA-E) that moderate a wide range of developmental responses, including germination, de-etiolation and phototropism. Phytochromes also affect adult plant architecture, regulate flowering time and are involved in circadian clock function (for review see Chen *et al.*, 2004). Mutant analysis has identified distinct yet often overlapping functions for phytochrome species, indicating that complex relationships between these molecules govern developmental processes. PhyA is primarily involved in responses to far-red light, with *phyA* null mutants failing to de-etiolate fully

when grown in these light conditions. PhyB is the major photoreceptor controlling de-etiolation under red light, and *phyB* null seedlings are characteristically elongated with unexpanded cotyledons under these conditions (Whitelam and Devlin, 1997). PhyB has perhaps the largest role of all the phytochromes, having central roles in germination, deetiolation, shade avoidance and flowering time. There is evidence of cross talk between phyC and phyA and B in the control of hypocotyl elongation, whilst phyD, E, and A act redundantly to control this response under red light (Franklin *et al.*, 2003b). phyC is also involved in cotyledon expansion under red light, with *phyAphyBphyDphyE* mutants retaining (albeit weakly) red/far red reversible induction of cotyledon expansion (Franklin *et al.*, 2003a; 2003b). Thus the integration of signals from different phytochromes allows fine control over a range of responses.

Phytochrome mediated responses may be grouped into three distinct modes of actions, low fluence responses (LFRs), very low fluence responses (VLFRs), and high irradiance responses (HIRs). The LFRs are the classical phytochrome mediated responses and are induced by R light, and may be reversed by a pulse of FR light. The effectiveness of FR in reversing the induction of these responses decreases, and is eventually lost as progressively longer dark intervals intersperse the R pulses. LFRs obey the law of reciprocity, whereby the duration of irradiance required to induce a response is inversely correlated to the intensity of light (reviewed in Mancinelli, 1994). Thus, LFRs may be induced by short pulses of high irradiance light, or long periods of low level illumination.

VLFRs are induced by very low photon fluences. Such responses are mediated by phyA, which is present in high levels in etiolated seedlings. These responses saturate at very low fluence rates and allow seeds to exploit transient light exposure during soil disturbances to germinate. VLFRs are genetically distinct from a second mode of action of phyA, the FR-

HIR, as distinct loci have been identified for each subset of responses. Furthermore, seedlings of the Columbia ecotype are severely impaired in the VLFR when compared to Landsberg (Yanovsky *et al.*, 1997). The third mode of response, the HIR, is triggered by prolonged FR treatments, with the extent of response being dependent on the irradiance and duration of the light treatment. Such responses are not R:FR reversible, and do not obey the law of reciprocity. *phyA* mutants are impaired in the FR-HIR, and maintain unexpanded cotyledons under such conditions (Whitelam *et al.*, 1993).

Phytochrome

Phytochromes are 124kDa protein dimers (Figure 1.1). The C terminal region of the phytochrome protein contains dimerization motifs, two PAS domains, and a histidine kinase related domain. PAS (PER-ARNT-SIM) domains are widely found in plant signalling networks and are important for protein-protein interactions. Such domains may also be important for signal or ligand stimulated responses. The N-terminus confers the spectral sensitivities of the molecule as the light sensing chromophore is covalently bound to a cysteine residue in this location (Fankhauser, 2001).

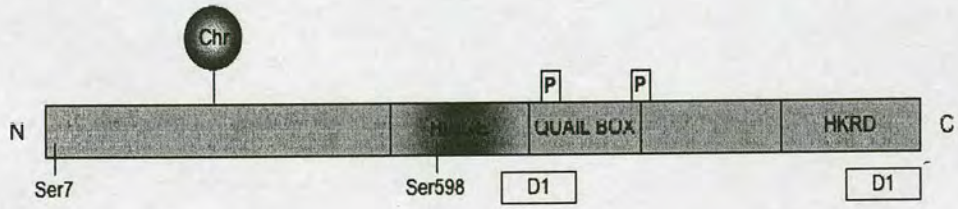


Figure 1.1: Phytochrome Structure
Phytochromes exist as dimers of 124kDa apoproteins, each bound to tetrapyrrole chromophore (Chr). Dimerization is facilitated by a pair of motifs in the C-terminal (D1, D2). A pair of PAS domains (P) are important for protein – protein interactions between phytochrome and other signalling molecules, whilst the Quail box contains regulatory sequences. Absorption of light causes isomerization in the chromophore and triggers changes throughout the phytochrome apoprotein.

The phytochrome chromophore, phytochromobilin, is a linear tetrapyrrole, and is synthesised in the plastid by an enzyme cascade from heme. The early stages of chromophore synthesis are shared with other tetrapyrrole containing substances, including chlorophyll. Later stages in the pathway that are specific to phytochromobilin synthesis have been determined through analysis of mutants with reduced levels of chromophore biosynthesis. One such mutant, *hy1* (long hypocotyl) has been shown to encode a heme oxygenase (AtHO1). This mutant maintains some, albeit low chromophore synthesis, which may reflect a degree of redundancy, as three other heme oxygenase sequences are present in the Arabidopsis genome (Muramoto *et al.*, 1999).

Light absorption by the chromophore results in isomerization between the two of the rings. This triggers autophosphorylation and conformational changes throughout the protein moiety. Thus, phytochromes exist as two isomers – a Pr form that absorbs maximally in red, and a Pfr form that absorbs in the far-red region of the electromagnetic spectrum. Phytochrome is synthesised in the Pr form, and upon absorption of red light is converted to Pfr, whilst application of far-red reverses this process. The process of photoisomerization gives phytochrome its unique signalling capabilities, as only the Pfr form is believed to be biologically active. In the natural environment this ability allows plants to perceive neighbouring competition. Under canopy shade, seedlings are exposed to a far-red enriched light, and consequently have a higher proportion of phytochrome in the Pr, or "off" state (Morelli and Ruberti, 2002; Franklin and Whitelam, 2005). This property is very useful experimentally as it allows experimental manipulation of active phytochrome levels. Providing white light grown seedlings with supplementary far-red, or end-of-day far-red treatments can significantly adjust the proportions of active and inactive phytochrome. In Arabidopsis there are two types of phytochrome. PhyA is the only example of a type I, or light labile phytochrome and is found at high levels in etiolated tissues. Following

conversion to the Pfr form phyA is rapidly degraded. The remaining four phytochromes are all type II, or light stable, and the Pfr form of these phytochromes are found abundantly in light grown tissues.

In The Light, Phytochromes Move To The Nucleus And Aggregate In Nuclear Foci

Recent studies have revealed that activation of phytochrome is accompanied by changes in the cellular location of the molecule (see Nagatani, 2004). Upon activation by light the phytochrome molecule undergoes a conformational change that exposes nuclear localisation signals in the PAS domain, thus facilitating its nuclear translocation (Chen *et al.*, 2005). This is thought to be important for phytochrome activity, and red light induced nuclear localisation of PHYB::GFP was shown to be far-red reversible (Kircher *et al.*, 1999). Several studies have revealed that in the nucleus phytochrome molecules aggregate in subnuclear foci (speckles), whilst speckling intensity has been shown to correlate with severity of response (Kircher *et al.*, 2002; Chen *et al.*, 2003). The precise function of the subnuclear speckling is not yet known, though it has been proposed as the site where phytochrome regulates down-stream signalling events such as transcription. Several lines of evidence are supportive of this view. Phytochrome has been shown to co-localise to subnuclear speckles with cry2 and with the transcriptional regulator, PIF3 (PHYTOCHROME INTERACTING FACTOR 3) (Mas *et al.*, 2000; Bauer *et al.*, 2004). Furthermore, the E3 ligase COP1 (CONSTITUTIVELY PHOTOMORPHOGENIC 1) recruits a positive regulator of photomorphogenesis, HY5 (LONG HYPOCOTYL 5) to subnuclear foci for degradation by the nucleosome, and COP1 has been shown to be essential for phytochrome-mediated destruction of PIF3 (Ang *et al.*, 1998; Hardtke *et al.*, 2000; Bauer *et al.*, 2004). Phytochrome regulation of nuclear gene expression is discussed in more detail below. It is not yet known whether phytochrome interacts with COP1 in subnuclear foci to control these events. Recent work has demonstrated that speckle formation is not essential for all phyB

responses, as biological activity has been demonstrated for phyB N-terminal dimers that localise to the nucleus, but do not form speckles, and for diffuse PHYB::GFP nuclear staining which occurs at low fluence rates of light (Chen *et al.*, 2003; Matsushita *et al.*, 2003).

Aggregations of phytochrome are not only found in nuclei. Cytoplasmic phyA aggregates into speckles known as sequestered areas of phytochrome, or SAPs (Hofmann et al., 1990). SAPs are thought to be important for proteolytic degradation of phytochrome, and may contain intermediates or products of ubiquitin mediated degradation (Speth et al., 1986, 1987).

Phytochrome Signalling Mechanism

The mechanisms by which phytochrome is able to transmit light information has received extensive attention in recent years. Mutant analysis and yeast-2-hybrid screening techniques have identified important signalling components, and a complex picture of phytochrome signalling has emerged. An overview of some of the mechanisms of phytochrome signal transduction is shown in figure 1.2, and is discussed in more detail below.

1. Direct Modification Of Transcription

The observation that phytochrome moves to the nucleus in the light led to the suggestion that phytochrome may directly regulate transcription. However, phytochrome is not able to directly bind DNA, and therefore this is not possible. Yeast-two-hybrid screening identified PIF3 (PHYTOCHROME INTERACTING FACTOR 3) as an interaction partner of both phyA and phyB. PIF3 binds to the Pfr form of the molecule. PIF3 is a member of the bHLH family of transcription regulators, and binds to the G-box elements found specifically in the

promoters of light regulated genes, including LHY (LATE ELONGATED HYPOCOTYL) and CCA1 (CIRCADIAN CLOCK ASSOCIATED). PIF3 is constitutively localised to the nucleus and thus its regulatory activities are dependent on photoconversion of phytochrome to Pfr, and associated nuclear localisation. Far-red light causes photoisomerization of the Pr form, and results in the dissociation of PIF3 from phytochrome. Phytochrome can therefore be thought of as the light regulated component of a transcriptional complex (Martinez-Garcia *et al.*, 2000).

Thus, PIF3 provides a direct link between phytochromes and gene regulation at the transcriptional level. Other members of the bHLH family of transcription factors are also known to have roles in light signalling. One of these, PIF4 has been identified as binding to the Pfr form of phytochrome, and regulates transcription through G-box motifs. However, unlike PIF3, PIF4 appears unable to bind both photoreceptor and promoter simultaneously. Consequently phytochrome interaction with PIF4 is proposed to repress transcription as it prevents PIF4 interaction with the G box. Other bHLH proteins with light signalling roles include HFR1 (LONG HYPOCOTYL IN FAR-RED LIGHT 1), PIL1 (PHYTOCHROME INTERACTING FACTOR-LIKE) and SPT (SPATULA), although these have been shown not to interact with phytochrome in co-immunoprecipitation experiments (Khanna *et al.*, 2004; Duek and Fankhauser, 2005). bHLHs are capable of homo and heterodimerisation, and therefore these and other bHLH proteins might interact with phytochromes indirectly through interaction with another member of the PIF family.

2. Indirect Regulation Of Gene Expression And Integration Of Light Signalling Pathways

Mutant screening for seedlings exhibiting a photomorphogenic phenotype in darkness has identified 11 loci. Eight of these *COP/DET/FUS* (*CONSTITUTIVELY*

PHOTOMORPHOGENIC/DE-ETIOLATED/FUSCA) loci have been shown to encode components of the COP9 signalosome. COP9 is a 19S regulatory particle, or "lid" of the 26S proteosome, and identifies and binds ubiquitinated substances within the cell, resulting in their degradation at the proteosome. COP1 is a COP/DET/FUS protein that does not encode a proteosome subunit and has been identified as an E3 ligase, specifically targeting positive regulators of photomorphogenesis, such as HY5 for labelling with ubiquitin (via E2 ubiquitin conjugating enzymes). *COP10* and *DET1* are two further *COP/DET/FUS* loci that do not encode COP9 subunits. COP10 and DET1 have recently been shown to act together, along with DDB1 (DAMAGED DNA BINDING PROTEIN 1) in a complex that enhances the activity of E2 ubiquitin conjugating enzymes (Yanagawa *et al.*, 2004). COP1, COP10 and DET1 therefore act together to repress photomorphogenesis by directing and enhancing the ubiquitination and degradation of positive regulators of photomorphogenesis.

GFP and GUS labelling experiments have shown that the cellular location of COP1 is regulated by light. COP1 accumulates at high levels in the nucleus of dark grown seedlings, and following transfer to light, nuclear levels of COP1 are greatly reduced. This is likely to be due to a direct interaction with a photoreceptor, and has been shown for two cryptochrome species, cry1 and cry2 (Yang *et al.*, 2001). Thus COP1 acts in the nucleus to suppress photomorphogenesis in the dark by targeting positive regulators for degradation. In the light, COP1 is removed from the nucleus and its repressive effects on photomorphogenesis are stopped. Expression of COP1-GFP in photoreceptor deficient backgrounds has indicated that COP1 nuclear localisation is regulated by each of the phytochromes, and also the cryptochromes. Thus COP1 appears to act as a point of integration for signals originating from many photoreceptors.

Yeast-two-hybrid screening identified HY5 as an interaction partner for COP1, and this was later confirmed using FRET (FLUORESCENCE RESONANCE ENERGY TRANSFER) (Andersson and Kay, 1998; Ang *et al.*, 1998). HY5 is a bZIP transcription factor and is constitutively located in the nucleus, where it promotes the expression of light regulated genes by interacting with G-box elements in promoter regions, therefore acting as a positive regulator of photomorphogenesis (Oyama *et al.*, 1997). HY5 is targeted for degradation by COP1 (Ang and Deng, 1994), and therefore is an important signalling component for multiple photoreceptors (Ang *et al.*, 1998; Wang *et al.*, 2001). Recently, HY5 has also been identified as a point of integration between light and hormone signals (Cluis *et al.*, 2004). Thus, HY5 is an important regulator of developmental responses in tune with the environment.

3. Phytochrome Signalling In The Cytoplasm

Although transfer of phytochrome to the nucleus correlates with the photoactivity of the molecule, a substantial proportion of both active Pfr and inactive Pr phytochrome is found in the cytoplasm in the light and/or the dark (around 30% of phyB) (Nagy and Schafer, 2002). Furthermore, physiological responses through changes in transcription are relatively slow (around 3 hours). There has therefore been great speculation as to whether phytochrome has a cytoplasmic role. In lower plants, such as mosses and ferns, physiological studies have indicated that photoreceptors are localised to, or associated with, the plasma membrane. This led to the proposal that the majority of phytochrome signalling occurred in the cytosol, and was further supported by microinjection experiments in the tomato *aurea* mutant, which is deficient in chromophore biosynthesis (Bowler et al., 1994; Neuhaus et al., 1997). These experiments indicated that at least some of the phyA and phyB signal cascades required activation of secondary messengers, including heterotrimeric G proteins. G proteins activate a bifurcated signal transduction pathway, modulating levels of cGMP and calcium, which in

turn promote expression of chalcone synthase (CHS) and development of chloroplasts respectively. G proteins were further implicated by pharmacological experiments in soybean (Romero and Lam, 1993). The involvement of G proteins is however controversial. Recent work by Jones *et al.,* (2003) disputes the link between phytochrome and G protein signalling, as mutants lacking either or both subunits of the G protein do not have altered sensitivity to red or far-red light.

4. Phytochromes As Light Regulated Kinases

Red and far-red light has been shown to control the levels of phosphorylation of nuclear and cytoplasmic proteins (Harter *et al.*, 1994), indicating that protein kinases and phosphatases are important regulators of light signalling. Indeed, phytochrome is itself a known phosphoprotein, and is readily labelled with ^{32}P *in vivo* (Biermann *et al.*, 1994). Two residues are phosphorylated, Ser7, in the N terminus, and Ser598, in the hinge region (Figure 1.1). Phosphorylation at Ser7 readily occurs in both the Pr and Pfr isomers, whereas phosphorylation of Ser598 occurs preferentially in the Pfr form. Ser598 may therefore be important for Pfr specific responses. Indeed phosphorylation at Ser598 has recently been shown to prevent the interaction of phytochrome with the early signalling components NDPK2 and PIF3 (Kim *et al.*, 2004). Experiments using purified preparations of oat phyA have showed phosphorylation of serine residues on the photoreceptor molecule itself, indicating that phyA had autophosphorylation activity. The exact function of, and residues affected by autophosphorylation remain unclear.

Interest in the potential kinase activity of phytochrome was heightened following the discovery that bacteriophytochromes act as light dependent kinase to function as sensor proteins in a two component signal system (Hwang *et al.*, 2002; Karniol and Vierstra, 2003). Light absorption by bacteriophytochrome results in autophosphorylation. Subsequent

dephosphorylation transfers the recently bound phosphate to a response regulator. Activation of the response regulator in this manner enables it to directly moderate transcription, or to regulate other cellular processes. Furthermore, protein sequence analysis reveals a histidine kinase related domain (HKRD) in the C terminal region. However, important residues within this domain are not conserved between the individual phytochrome species, and Krall and Reed (2000) showed that although the HKRD was necessary for a full complement of phyB responses, it was dispensable. Subsequently, it has been shown that recombinant PHYA lacking the HKRD maintains kinase function *in vitro* (Kim *et al.*, 2005). The HKRD has been shown to interact with PKS1 (PHYTOCHROME KINASE SUBSTRATE 1), and may therefore be important for other aspects of phytochrome signalling. Although the HKRD may not be important for kinase activity, phytochrome purifications do exhibit kinase activity *in vitro*, though unlike bacteriophytochromes, plant phytochromes act as serine/threonine kinases. Several substrates of phytochrome kinase activity have now been identified, including PKS1, ARR4 (ARABIDOPSIS RESPONSE REGULATOR 4), the cryptochromes and the Aux/IAA proteins, suggesting that phytochrome kinase activity may be important in the integration of hormone and light information (Hamada *et al.*, 1996; Fankhauser *et al.*, 1999; Reed, 1999; Colon-Carmona *et al.*, 2000). The exact role of phytochrome kinase activity remains unclear, but the isolation of PKS1 and ARR4 by yeast-2-hybrid screening has provided room for speculation. As mentioned above, PKS1 binds to the HKRD, and is phosphorylated in a light dependent manner by phyA. PKS1 is a negative regulator of phytochrome signalling, with loss of function alleles affecting the VLFR of phyA. PKS1 is constitutively cytoplasmic, and its interaction with both phyA and phyB has been proposed to anchor both these phytochromes in the cytoplasm, thereby preventing their transfer to the nucleus (Fankhauser *et al.*, 1999). Isolation of ARR4 suggested that plant phytochrome could act like bacteriophytochrome in a two component system (Sweere *et al.*, 2001). However, ARR4 is a specific component of

phyB signalling, and kinase activity has yet to be shown for phytochromes other than phyA. Thus, both cytosolic retention and two component receptor functions for phytochrome kinase activity remain hypothetical (Fankhauser, 2001; Nagy *et al.*, 2001).

Phytochrome and Auxin Signals Interact to Coordinate Development

The phytohormone auxin has wide ranging effects on plant development. Auxin is a regulator of many cellular responses, including cell division, expansion and differentiation, and is therefore able to regulate organ patterning, tropisms and plant architecture. Auxin is synthesised in the shoot apex and developing leaves and is subsequently transported through the tissues and vasculature downwards towards the root tip. Transport of auxin occurs in a controlled manner, via polar auxin transport (PAT), enabling fine tuned distribution within tissues. PAT is an essential characteristic of auxin action. Several reports have indicated that auxin and phytochrome signalling are intricately linked. End-of-day far-red (EOD-FR) light treatments (which deplete phytochrome at the end of the day) result in hypocotyl elongation and trigger the expression of auxin inducible genes (Tanaka *et al.*, 2002b). Stabilisation of IAA3, a negative regulator of auxin signalling in the *shy2-2* mutant leads to a photomorphogenic phenotype in darkness (Kim *et al.*, 1998). Furthermore, light regulates phototropism and gravitropism, at least partly through asymmetric redistribution of auxin. Thus, auxin and phytochrome signalling pathways intercept at many points, allowing tight control over growth and development. The molecular basis of these multiple integration points is discussed below.

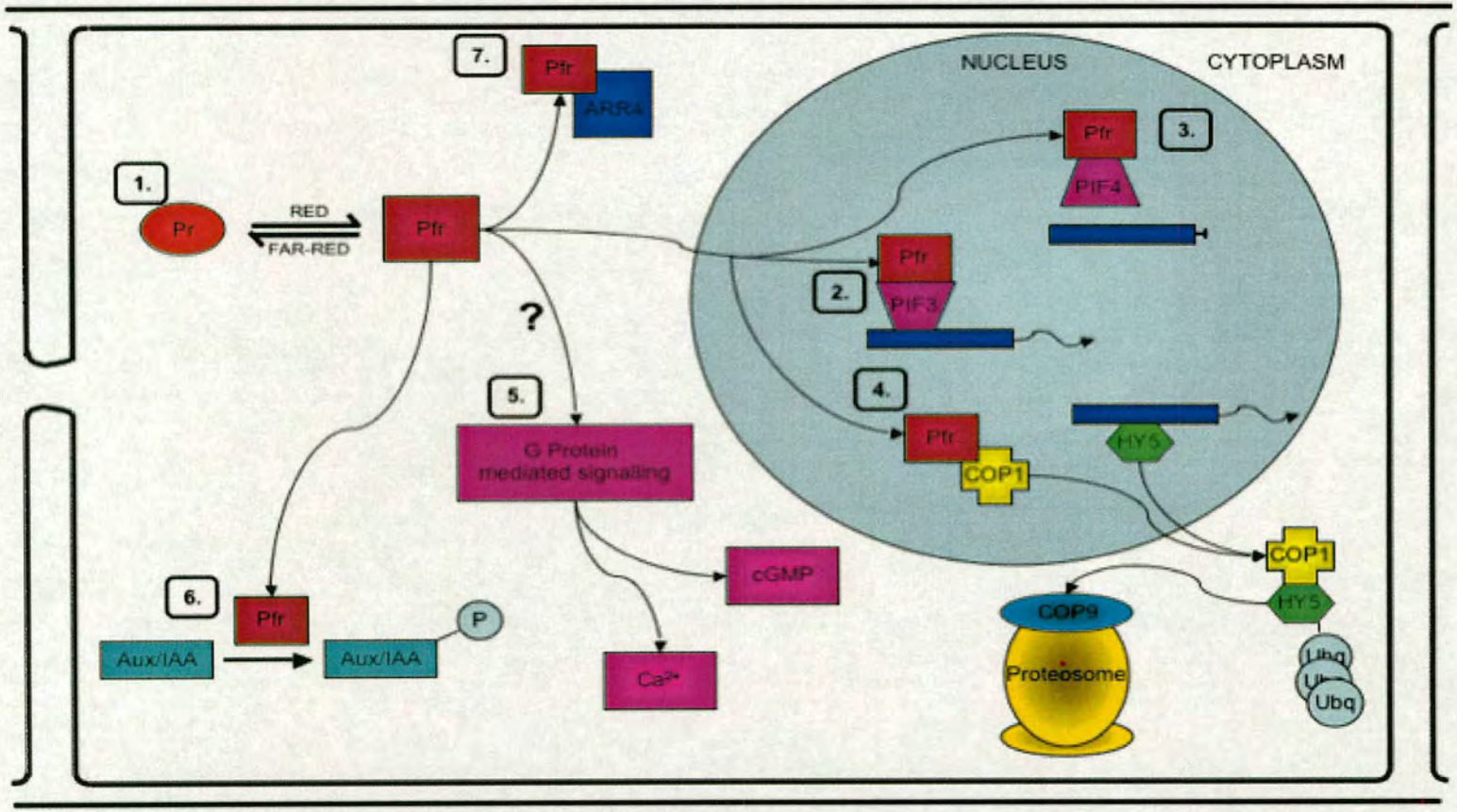


Figure 1.2: Mechanisms of phytochrome mediated signal transduction.
1. Absorption of red light promotes photoconversion to the Pfr form, revealing nuclear localization signals. Phytochrome moves to the nucleus where it is able to directly interact with transcription factors, including PIF3 (2.) and PIF4 (3.) to regulate gene expression. Pfr phytochrome also interacts with the E3 ubiquitin ligase COP1 (4.), thereby removing it from the nucleus. In darkness, COP1 in the nucleus labels positive regulators of photomorphogenesis, such as HY5 with ubiquitin (ubq), thereby targeting them for degradation at the 26S proteosome. Removal of COP1 from the nucleus therefore releases repression of HY5, a bZIP transcription factor, and gene expression begins. Phytochrome may have roles outside of the nucleus, including regulation of G protein signaling, including Ca^{2+} and cGMP signaling (5.), and may signal by catalyzing phosphorylation events. Kinase substrates of phytochrome include ARR4, possibly indicating that plant phytochrome, like bacteriophytochrome may act as part of a two component signaling pathway. PhyA is also known to phosphorylate members of the Aux/IAA family, indicating that phytochrome kinase activity may be an important regulatory step in auxin signaling.

1. Auxin Homeostasis

RED1 was initially isolated as a phytochrome signalling component, with *red1* seedlings exhibiting reduced photomorphogenesis specifically under red light (Wagner *et al.*, 1997). *RED1* was subsequently shown to be allelic to *ATR4/SUR1*, and to encode CYP83B1, a cytochrome P450 monooxygenase. This enzyme catalyses hydroxylation of the IAA precursor indole-3-acetaldoxime (IAOx), and its inhibition results in an accumulation of IAA as the pool of IAOx available for IAA synthesis is increased. Thus RED1 provides a mechanism for phytochrome mediated control of auxin biosynthesis (Hoecker *et al.*, 2004)

2. Polar Auxin Transport

Several studies have implicated phytochrome as a regulator of polar auxin transport. Application of the auxin transport inhibitor NPA (N-1-napthylthalamic acid) to light grown seedlings reduces hypocotyl elongation, but such repression is not observed when NPA is applied to etiolated seedlings (Jensen *et al.*, 1998). Furthermore, the magnitude of this response is reduced in *phyA*, *phyB* or *cry1* seedlings grown under far-red, red or blue lights respectively (Jensen *et al.*, 1998). Thus, it seems that inhibition of hypocotyl elongation in the light is mediated at least partially by photoreceptor regulation of auxin transport.

Directional auxin flow, via PAT, is dependent on polarly localised PIN auxin efflux regulators (Blakeslee *et al.*, 2005). The positioning of PIN proteins is itself regulated by auxin which regulates the cycling of PINs between endosomes and the plasma membrane (Paciorek *et al.*, 2005). A large calossin-like protein, BIG, appears to be involved in this process, and thereby intimately involved in PAT. Mutant alleles of BIG (*tir3/doc1/asa1/umb1*) not only have decreased PAT, but also exhibit altered photomorphogenic traits (Li *et al.*, 1994; Gil *et al.*, 2001; Kanyuka *et al.*, 2003).

tir3/doc1/asa1/umb1 mutant alleles do not display normal elongated hypocotyl phenotype in darkness, and are consequently shorter than wild type seedlings. Microarray analysis revealed that several light- regulated genes were activated in dark grown *doc1* seedlings. Interestingly, the expression of these genes is suppressed by increasing auxin levels. Analysis of BIG therefore illustrates that normal auxin transport is essential for the growth of etiolated seedlings in the dark. Disruption of this process interferes with the dark – light developmental switch.

Further evidence for a link between phytochrome and auxin transport comes from analysis of ATHB-2. *ATHB-2* encodes a homeodomain-leucine zipper transcription factor, and its expression is rapidly upregulated in response to low R:FR ratio light (simulated shade) (Carabelli *et al.*, 1993). This increase in expression has subsequently been shown to be regulated by phyB and phyE (Franklin *et al.*, 2003b). Suppression of *ATHB-2* (using antisense overexpression) results in an enhanced de-etiolation phenotype, with seedlings exhibiting a shorter hypocotyl and enlarged cotyledons. Conversely seedlings overexpressing *ATHB-2* resemble phytochrome loss of function mutants (Steindler *et al.*, 1999). Thus, ATHB-2 is a regulator of the shade avoidance response. Overexpression of ATHB-2 not only affects shoot development, and has also been shown to reduce the production of lateral roots. Lateral root growth is promoted by auxin from the shoot, leading to the hypothesis that the *ATHB-2ox* lateral root phenotype was a result of decreased auxin flow from the shoot to the root (Morelli and Ruberti, 2002). Indeed, lateral root production in *ATHB-2ox* can be restored by application of exogenous auxin. ATHB-2 is therefore proposed as a component in phytochrome regulation of auxin transport (Morelli and Ruberti, 2000).

3. Signalling components shared by light and auxin

Auxin signalling is mediated through transcriptional regulation of three gene families: *GH3*-related genes, *Aux/IAAs* and *SAURs* (small auxin upregulated RNAs). Light has been shown to regulate transcription and/or influence the activity of auxin-regulated genes (Abel *et al.*, 1995; Devlin *et al.*, 2003; Tepperman *et al.*, 2004).

The GH3 family of Arabidopsis consists of 20 genes. At least 6 of these act as IAA-amido synthases that catalyse the conjugation of amino acids to, and inactivation of, IAA (Staswick *et al.*, 2005). Thus GH3s are important regulators of auxin homeostasis. Several members of the GH3 family exhibit altered photomorphogenic traits, implicating *GH3* mediated conjugation of IAA as a point of moderation of auxin signalling by light.

dfl1-D (GH3-6) gain of function mutants have short hypocotyls under red, far-red and blue lights, indicating that DFL1 is involved in light specific inhibition of hypocotyl elongation and is under the control of phytochromes and cryptochromes (Nakazawa *et al.*, 2001). Like DFL1, *DFL2ox* (GH3-10) also enhances light regulated inhibition of hypocotyl elongation, and is thought to be downstream of light stable phytochromes. However, these genes have very different expression characteristics (Takase *et al.*, 2003). Whilst *DFL1* transcription is regulated by auxin and not light, *DFL2* is light and not auxin regulated. Thus, regulation of DFL1 by light may occur post-transcriptionally, and indicates that different GH3 family members are regulated via different mechanisms.

As well as different mechanisms of control, auxin and light may have very different effects on gene family members. YDK1, another GH3 family protein, for example, is positively regulated by auxin, and negatively regulated by blue and far-red light. Overexpression of *YDK1* alters the phenotypes of both light and dark growth seedlings. Light may regulate

YDK1 expression indirectly through moderation of auxin via previously described mechanisms, or *YDK1* may be under dual control by both auxin and light (Takase *et al.*, 2004). Transcription of a fourth member of this gene family, GH3α is elevated in response to EOD-FR, or depleted *phyB*. However, phyB regulation of GH3α is not sustained in *axr2-1/iaa7* gain of function mutants, indicating that normal auxin signalling is required for this phyB mediated response (Tanaka *et al.*, 2002a). Thus, members of the GH3 family are subject to control by auxin and light via different mechanisms, highlighting the complexity of the interaction between these two signalling networks.

The *Aux/IAAs* are a second family of auxin-regulated genes. In Arabidopsis there are 28 *Aux/IAAs* that operate by binding to, and negatively regulating the ARF family of transcription factors. Auxin controls *Aux/IAA* levels by stimulating ubiquitin mediated proteolysis of these proteins via the ubiquitin ligase SCF^{TIR1} (Kepinski and Leyser, 2004). TIR1 is an F-Box protein and component of the SCF complex that targets *Aux/IAAs* for ubiquitination and subsequent degradation. Auxin promotes the interaction between TIR1 and Aux/IAAs. SCF^{TIR1} mediated degradation of Aux/IAAs feeds back to regulated Aux/IAA transcription, and newly synthesised Aux/IAAs rapidly restore repression of ARFs (Kepinski and Leyser, 2005). This leads to a highly dynamic system that is very responsive to changes in inputs.

Aux/IAA turnover is important for aspects of light regulated development. Indeed, mutations that stabilize IAA3 (SHY2) were originally isolated as suppressors of *hy2* and *phyB* phenotypes. *shy2-2* gain of function mutants have a photomorphogenic phenotype in the dark, with a short hypocotyl and expanded cotyledons, and elevated levels of *CAB* mRNA (usually repressed in dark grown seedlings) (Kim *et al.*, 1998). A short hypocotyl and expanded cotyledons are also observed in dark grown seedlings of gain of function

mutants of two other *Aux/IAAs* – *axr2/iaa7*, and *axr3/iaa17* (Leyser *et al.*, 1996; Nagpal *et al.*, 2000). This suggests that normal turnover of Aux/IAAs is important for repressing photomorphogenesis in dark grown seedlings. The mechanism by which light is able to regulate Aux/IAA activity is not yet known. Several *Aux/IAAs* are transcriptionally regulated by both phyB and phyA (Devlin *et al.*, 2003), and interestingly *in vitro* studies have demonstrated that oat phyA is able to phosphorylate Aux/IAAs, and that SHY2 can interact with phyB (Colon-Carmona *et al.*, 2000; Tian *et al.*, 2003). Thus phytochromes may regulate Aux/IAA activity either at the transcription or post-translational levels, further highlighting the complexity of auxin light interactions.

Phytochrome Regulation of Phototropism

Positive phototropism of plant stems was first described by Darwin in 1896 (reviewed in Orbovic and Poff, 1993). The photoreceptors responsible for this movement are the phototropins (phot1 and phot2), and their effects are moderated by the phytochromes and cryptochromes (Liscum and Stowe-Evans, 2000; Stowe-Evans et al., 2001; Briggs and Christie, 2002). Early investigations into phototropism led to the formulation of the Cholodny-Went hypothesis and identification of auxin (reviewed in Liscum, 2002). The Cholodny-Went hypothesis states that a phototropically stimulated shoot bends towards the stimulus due to an increase in auxin at the shaded side of the stem, and consequently increased cell elongation. Such an auxin gradient is now known to be the product of polar auxin transport, and two PIN proteins are known to be important – PIN1 and PIN3. PIN1 delocalisation from the basal cell wall in phototropically stimulated stems is PHOT1 dependent, and absent in *phot1* mutants (Blakeslee *et al.*, 2004). PIN3 is also essential for the establishment of the auxin gradient and is known to be regulated by phyA and phyB (Friml, 2003). Furthermore, Lariguet and Fankhauser (2004) showed that phyA is an important regulator of phototropism in response to blue light, and that it acts by suppressing

the tropic response to gravity. Further indication of the involvement of phytochromes in moderating phototropism comes from the observation that phototropic response to blue light may be enhanced by previous exposure of seedlings to red light, and mutant analysis has indicated that this is mediated by phyA, and to a lesser extent phyB.

Arabidopsis roots also exhibit phototropic response to blue light, though move away and not towards a light source. Roots do however exhibit positive phototropism to red light. Such responses are weak, and only revealed in the absence of gravity. Analysis of mutants lacking each of the phytochrome species indicates that these responses are mediated by phyA and phyB (Kiss *et al.*, 2003a). Though the mechanisms underlying root phototropic responses are unknown, it is assumed that they utilise mechanisms similar to those of the shoot.

Phytochrome regulation of gravitropism

As in phototropism, responses to gravity are generated by asymmetric gradients of auxin. The mechanism of perception of the gravity stimulus remains unclear. Starch statoliths within specialised cells (statocytes) were thought to be involved, as they are displaced with changes to the gravity stimulus. However, although impaired in gravity response, starchless and plastid deficient mutants are not complete devoid of gravity responses, indicating this is not the only method of gravity perception (Kiss *et al.*, 1989; Kiss and Sack, 1989). Statolith sedimentation appears to activate actin dependent relocalisation of PIN3. PIN3, like PIN1, cycles between the endosomes and the plasma membrane. As statoliths are enmeshed in actin, sedimentation results in rearrangement of the cytoskeleton, and PIN3 is consequently relocalised to the side of the cell (Friml *et al.*, 2002). Auxin is then redirected, and the shoot or root bends accordingly. Like phototropism, the mechanisms for shoot and root gravitropism remain uncertain, but are postulated to share at least some of the same

mechanism, though isolation of *sgr* (shoot agravitropic) mutants indicates that at least some components are unique to the shoot (Yano *et al.*, 2003; Morita and Tasaka, 2004).

Dark grown seedlings exhibit strong negative gravitropism, whereas red and far-red grown seedlings grow in random orientations, indicating that these wavelengths of light act to reduce the gravity response in young seedlings. phyA and phyB are important modulators of this response, with phyA also regulating gravitropism in blue light (Liscum and Hangarter, 1993; Hangarter, 1997). Like phototropism, this may involve regulation of polar auxin transport, PIN3 again being a potential point of integration (Friml *et al.*, 2002). A further class of auxin transport proteins may also be involved. The involvement of MDR1 (MULTI-DRUG RESISTANT 1) and PGP1 (P-TYPE GLYCOPROTEIN 1) in gravitropism and auxin transport has been demonstrated (Noh *et al.*, 2003), and these genes are light regulated. *PGP1ox* results in hypocotyl elongation, specifically in the light, and *MDR1* expression is reduced in the light.

Root gravitropism is also affected by light. Recent work by Correll and Kiss (2005) demonstrated that gravity responses of *phyB*, and *phyAphyB* mutants was attenuated in both primary and lateral roots. Interestingly the *phyAphyB* double mutant phenotype is similar to that of mutants lacking the transcription factor *hy5*, which is known to be downstream of multiple photoreceptors, including phyA, phyB and cry1 (Oyama *et al.*, 1997). This mutant is known to have altered auxin signalling, including down-regulated gene expression of several *Aux/IAA* genes including *AXR2/IAA7* (Cluis *et al.*, 2004). Thus, HY5 is a likely point of integration of multiple light signals with auxin.

Phytochromes Have Roles In Roots

Whilst the role of phytochromes in the shoot has been extensively studied, little attention has been directed at the role of these photoreceptors in root development. However, there is evidence for phytochrome activity within the Arabidopsis root system. Roles have been identified for phytochromes in the control of phototropism in roots (Ruppel *et al.*, 2001; Correll *et al.*, 2003; Kiss *et al.*, 2003b). This work demonstrated that both primary and lateral roots exhibit positive phototropism in response to red light, and that this response is lost in the primary roots of *phyA* and *phyB* seedlings. PhyD and phyE were also shown to be involved in these responses, but their roles were only minor. Studies using transgenic plants expressing *PHYp::GUS* or *PHYp::LUC* have provided evidence for phytochrome expression in roots (Somers and Quail, 1995; Goosey *et al.*, 1997; Hall *et al.*, 2001; Toth *et al.*, 2001). Thus, roots clearly have the ability to respond to directional light, though this response is largely overridden by the stronger gravitropic response. However, as phytochrome has also been implicated in root gravitropism this provides a mechanism for interplay between these two pathways (Correll *et al.*, 2003).

The role for phytochromes in roots does not appear to be confined to the tropic responses. Early work by Reed and co-workers (1993) demonstrated a role for phyB in the control of root hair elongation. More recently, phytochromes A, B and D have been shown to control red light-mediated elongation of the primary root (Correll and Kiss, 2005). The *hy5* mutant, known to be defective in phytochrome signalling, also has a striking pleiotropic root phenotype. *hy5* which has a long hypocotyl phenotype, also produces longer root hairs than wild type and exhibits an altered rate of lateral root production and reduced gravitropism(Oyama *et al.*, 1997; Cluis *et al.*, 2004). HY5 has been shown to control these aspects of root growth by altering signalling through the cytokinin and auxin pathways

(Cluis *et al.*, 2004). Thus, HY5 has been proposed as a signal integration point between the light and hormone signalling networks.

Long Distance Signal Transduction

Synchronisation of development in response to environmental cues requires transmission of information between individual cells, and between different tissues. There are several examples of root to shoot communication for coordinated development, including responses to drought and cadmium stresses (Sauter *et al.*, 2001; Gong *et al.*, 2003). Recent work by Turnbull *et al.*, (2002) using micrografting techniques has revealed a role for root derived signals in the control of shoot branching. Whilst hormones play an important role in signal transduction over long distances (Sauter *et al.*, 2001), signalling roles for many small molecules have been identified, including NO_3 and CO_2 (Lake *et al.*, 2002; Takei *et al.*, 2002). However, little is known about synchronisation of root development to information perceived by the shoot. Light information plays a significant role in regulating shoot development (Whitelam *et al.*, 1998; Casal *et al.*, 2003), and it is logical that such extensive changes in shoot development require coordinated responses from the root.

A Root Focussed Approach to Understanding Phytochrome Signalling

Light information, acting through the phytochromes, plays an important role in regulating shoot development. Though phytochromes are known to be present in root tissues (Goosey *et al.*, 1997; Toth *et al.*, 2001), and have been reported to regulate phototropism in roots (Correll *et al.*, 2003; Kiss *et al.*, 2003a), little is known about how they regulate root growth. Furthermore, HY5, an important component of light signal transduction, is known to regulate root development. I was therefore interested in establishing the extent to which phytochromes were involved in regulating root development, and was interested to know if

phytochromes could act locally as a shoot derived, transmitted signal to regulate development. As auxin is intimately involved in root responses, I was also keen to understand whether phytochrome and auxin signalling are also interconnected in roots. In this study, I address these issues, using phytochrome null mutants and reporter genes to dissect phytochrome action, and show that phytochromes are in fact important regulators of root growth, acting collectively to exert tight control over root phenotypes. I show that shoot phytochromes are able to act over long distances to regulate the emergence of lateral root development, at least partly via a shoot derived auxin pulse. My work shows that the phytochromes regulate the elongation of root hairs, and that *phyB* interacts genetically with *shy2-2* to control this response. I also provide evidence that phytochrome is able to act in the Pr form to regulate root responses. In this thesis, I identify new roles for phytochrome and develop an exciting new approach to understanding phytochrome signalling.

Chapter 2

Integration of Phytochrome and Auxin Signals in Roots

An emerging lateral root of a seedling expressing *DR5::GUS*

CHAPTER 2

INTEGRATION OF PHYTOCHROME AND AUXIN SIGNALS IN ROOTS

INTRODUCTION

In plants, sophisticated signalling pathways have evolved to interconnect sensory input and developmental pathways. These signalling networks ensure that development is synchronised with local and seasonal environmental changes, and that it proceeds in a timely and co-ordinated fashion. Hormones, such as auxin, play an integral part in these signal networks, transferring information from environmental stimuli to downstream effectors causing developmental changes. Auxin and light signalling pathways are highly integrated in the shoot, but their interactions in the root are less well understood.

In the shoot, the transcription factor HY5 has been proposed as an important integrator of light and hormone signals (Cluis et al., 2004). *hy5* mutants have elongated hypocotyls and are known to be defective in phytochrome signalling. *hy5* mutants also have a striking pleiotropic root phenotype, with altered root gravitropism and rate of lateral root emergence (Oyama et al., 1997; Cluis et al., 2004). Lateral roots are branches off the main root that increase a plants capacity for nutrient extraction and water use efficiency, as well as increasing plant anchorage (Grierson and Ketelaar, 2004). Coordination of lateral rooting with seedling development is therefore very important for optimising plant growth.

Lateral roots originate from the root pericycle, distal to the primary elongation zone (Dubrovsky et al., 2000). In lateral root founder cells, reactivation of the cell cycle results in

the establishment of a meristem (Celenza et al., 1995; Malamy and Benfey, 1997). Pairs of founder cells, opposite to the xylem pole, divide asymmetrically giving rise to an inner and outer layer of cells (Dolan et al., 1993; Malamy and Benfey, 1997) and further transverse divisions of both layers forces the developing lateral root into the cells of the cortex. Successive divisions force the lateral root through the epidermis.

Lateral root emergence is strongly affected by environmental cues. Nutrient availability is one such cue. Application of nitrate, for example, can both stimulate and inhibit lateral root elongation, depending on the concentration used (Zhang et al., 1999). The hormones ABA and cytokinin have been implicated (De Smet et al., 2003; Rani Debi et al., 2005), and auxin has been identified as a key regulator of lateral root emergence. Pharmacological and genetic analyses have shown that auxin is important at several specific developmental stages.

Analysis of mutants with impaired lateral root production has revealed altered auxin responses. *superroot* mutants, for example, produce excessive lateral roots and have elevated levels of free IAA (Celenza et al., 1995). Several mutants with altered auxin responses are also affected in lateral root production (for review see Casimiro et al., 2003), including *axr4* (auxin resistant) seedlings which have reduced lateral rooting (Hobbie and Estelle, 1995). Kerk and Feldman (1995) provided further evidence for a link between auxin and lateral root emergence by adding radiolabelled auxin to seedlings and showed an accumulation of this hormone in developing lateral root primordia.

The initiation of lateral root primordia is determined by a basipetal auxin gradient. This gradient is established, albeit weakly, following germination (Bhalerao et al., 2002) through combined action of auxin influx and efflux carriers. Seedlings lacking the *aux1* auxin influx carrier are unable to establish this gradient, and consequently have reduced IAA

concentrations at the root tip. Due to the absence of the auxin gradient, *aux1* seedlings produce 50% fewer lateral root primordia than wild type seedlings (Swarup et al., 2001; Marchant et al., 2002). The importance of a basipetal auxin gradient in the root has been further demonstrated using the auxin transport inhibitor *N*-1-napthylphthalamic acid (NPA). Inhibition of auxin transport through local application of NPA to the root prevents the development of lateral root primordia in a dose dependent manner (Reed et al., 1998; Himanen et al., 2002), and results in accumulation of auxin at the root tip resembling that observed in *aux1* mutants (Casimiro et al., 2001). The inhibitory effects of NPA may be released by transferring seedlings to control or auxin rich medium, and results in a rapid increase in auxin in the pericycle (visualised using the auxin responsive promoter reporter gene fusion, *DR5:GUS*) (Ulmasov et al., 1997), and a recovery in the production of lateral roots (Casimiro et al., 2001).

Further inspection of the pericycle reveals that the production of lateral root primordia is prevented by locking these cells in G1 phase of mitosis (Himanen et al., 2002). Auxin therefore stimulates the production of lateral root primordia in the pericycle through reactivation of the cell cycle (Casimiro et al., 2003). Little is known about how the spacing of lateral roots is determined, though auxin regulated proteins such as SINAT5 have been implicated (Xie et al., 2002).

The establishment of local auxin gradients within the root is therefore important for the establishment of lateral root primordia. However, auxin has wide ranging effects on lateral root development. Wightman and Thimann (1980) identified the importance of shoot derived auxin in the production of lateral roots. They removed cotyledons of pea seedlings and observed a reduction in the number of lateral roots produced. Application of auxin to the cut site was able to partially recover lateral root production (Wightman and Thimann,

1980). Similarly, in *Arabidopsis,* excision of the shoot results in a 4 fold decrease in the production of lateral roots (Bhalerao et al., 2002), and may be rescued by supplemental application of IAA (Reed et al., 1998). Shoot derived auxin is therefore implicated in the production of lateral roots.

Recent work by Bhalerao *et al.,* (2002) showed that the emergence of the first lateral root primordia correlated with a transient increase of auxin. Excision experiments identified that a pulse of auxin originated from the first true leaves, 5-7 days after germination. Removing the apical parts of the plant prior to this pulse prevented the emergence of lateral roots but did not affect the number of lateral root primordia. Removing the shoot subsequent to this pulse had no effect on the emergence of lateral roots. Thus it appears that transient augmentation of root auxin, via a shoot-derived pulse early in seedling development is essential for lateral root elongation.

Thus it has been identified that auxin is required at multiple developmental stages. Designation of lateral root primordia is dependent on a basipetal auxin gradient that is established early in development, whereas elongation of lateral roots is initially dependent on a shoot derived auxin pulse. By 10 days post germination, the root system has acquired sufficient capacity to synthesise auxin, predominantly in the primary and lateral root tips, and becomes largely independent of shoot derived auxin (Ljung *et al.*, 2005). Lateral roots develop the capacity to act as auxin sources between developmental stages III – IV (Casimiro et al., 2003).

Auxin is therefore intimately involved in lateral root development. Given the tight linkages between phytochrome and auxin signalling in the shoot, I was interested to determine

whether phytochrome and auxin signalling are interconnected in roots, and whether phytochromes were therefore able to affect lateral root emergence.

RESULTS

Phytochrome Null Mutants Have Altered Rates of Lateral Root Emergence

Given the crucial role of auxin in lateral root emergence (Casimiro et al., 2003) and the growing evidence of a link between phytochrome and auxin signalling (Colon-Carmona et al., 2000; Tian et al., 2003), I was keen to establish whether phytochrome had a role in controlling lateral root production. For this analysis, I grew seedlings null for one or more phytochrome species on vertically orientated plates under light of 100μmol $m^{-2}s^{-1}$ photon fluence rate. I counted lateral root emergence in each genotype between 7 and 11 days post induction. *hy5* null mutants were used as a control, as the role of HY5 in lateral root emergence has been characterised (Oyama et al., 1997; Cluis et al., 2004). In my experiments, young *hy5* seedlings produced more lateral roots than wild type seedlings, the rate falling off after day 10 such that wild type and *hy5* seedlings had produced similar numbers of lateral roots by day 11 (Figure 2.1a). These results are consistent with previous reports, but pinpoint more precisely the timing of deceleration in *hy5* lateral root emergence, which occurs after day 9 (Cluis et al., 2004). My results provide evidence that the phytochromes moderate lateral root emergence (Figure 2.1a). When compared to wild type seedlings, *phyA* and *phyE* mutants exhibited similar responses, with both mutants producing fewer lateral roots. This response was even more perturbed in the *phyB* null mutant, which produced even fewer lateral roots than *phyA* or *phyE*. These data suggest that phyA, phyB and phyE are all positive regulators of lateral emergence, with phyB in the most influential role. Interestingly, my analyses revealed an opposing role for phyD in the control of lateral root emergence. *phyD* mutants produced more lateral roots than wild type, with similar numbers to *hy5,* though unlike *hy5* the rate of lateral root production did not decrease between days 9 and 10. These data suggest a role for phyD as a negative regulator of lateral root emergence.

To establish the genetic relationship between individual phytochrome species in the control of lateral root emergence rate, I assessed mutants lacking one or more phytochromes in addition to phyB. As indicated above, lateral root production was reduced or more severely perturbed in the *phyA* and *phyB* mutants, respectively. The combined effect of these mutations, however, led to an intermediate phenotype (Figure 2.1b). This indicates that the phyA and phyB signalling pathways interact to control lateral root emergence. In contrast, the *phyBphyD* double mutant produced fewer lateral roots than the wild type at a rate that closely resembled that of the *phyB* monogenic mutant (Figure 2.1c). Thus, removal of *phyB* appears to overcome the *phyD*-induced enhancement of lateral root emergence. These data illustrate that *phyB* is epistatic to *phyD* for this response, suggesting phyB is required for the *phyD* monogenic mutant phenotype. In contrast, the *phyBphyE* mutant initially produced lateral roots at a similar rate to the wild type, however this slowed after 9 days (Figure 2.1c). This phenotype resembles that of the *phyE* monogenic mutant, suggesting that *phyE* is at least partially epistatic to *phyB* for this response. The *phyBphyDphyE* triple mutant exhibited a lateral emergence phenotype that was intermediate between the *phyBphyD* and the *phyBphyE* double mutants (Figure 2.1c). As the monogenic mutant analysis suggests opposite roles for phyD and phyE (Figure 2.1a), this indicates that these phytochromes are likely to act in separate pathways in this response. Collectively my data illustrate a high level of complexity in phytochrome control of lateral root emergence.

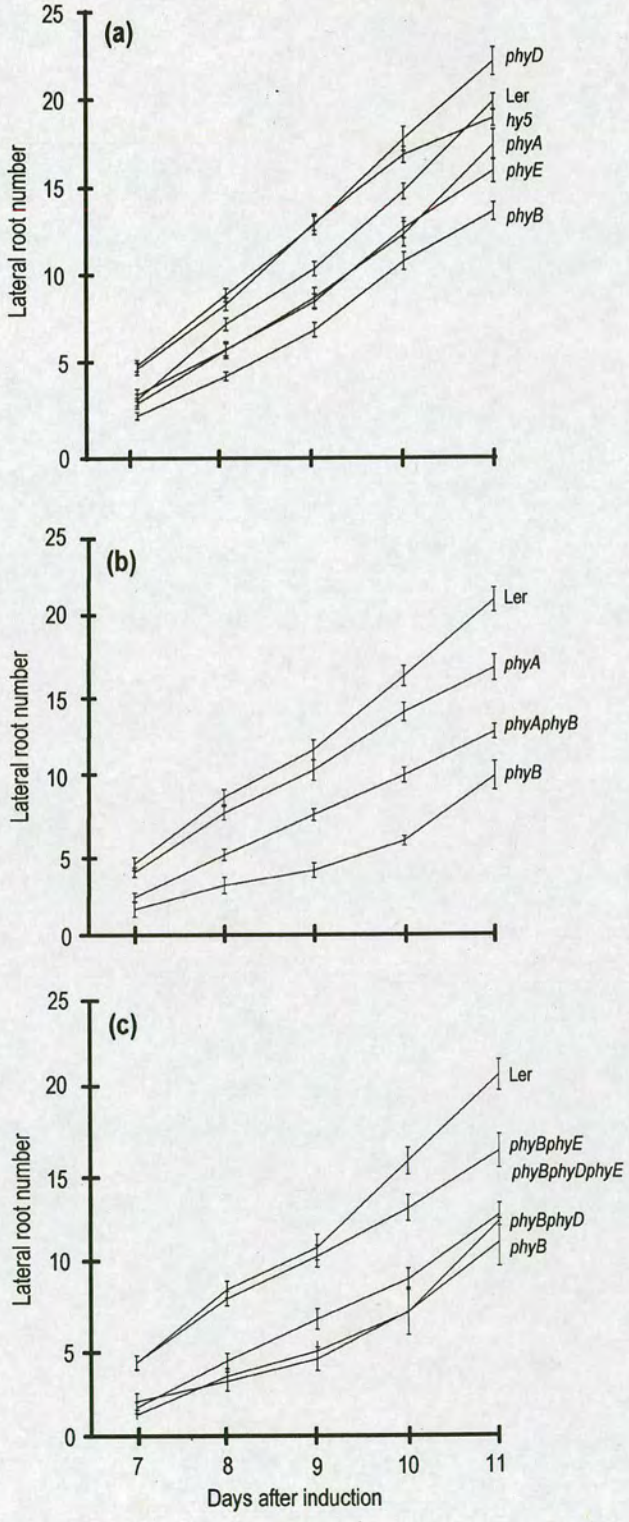


Figure 2.1: Lateral emergence in phytochrome null mutant seedlings.
Lateral root number of Ler (WT), *phyA, phyB, phyD, phyE* and *hy5* (a); Ler (WT), *phyA, phyB, phyA, phyB* and *phyAphyB* (b); and Ler (WT), *phyB, phyBphyD, phyBphyE,* and *phyBphyDphyE* (c). Seedlings were grown under 16h photoperiods at 18°C. Data represent mean values from at least 30 seedlings. Standard error bars are shown.

Low R:FR ratio light reduces lateral root emergence rate

It is now well established that phyB has a major role in controlling de-etiolation. The other phytochromes all contribute, albeit in more minor ways to this response (Franklin and Whitelam, 2004). Thus, seedlings that are null for phyB or for one or more phytochromes in addition to phyB fail to de-etiolate fully under red or white light. These seedlings are all impaired in hypocotyl inhibition and cotyledon expansion. A similar phenotype is observed in seedlings where active phytochrome levels are depleted in response to low R:FR ratio light (Robson *et al.*, 1993). My analysis of seedlings null for phytochrome species has revealed a complex relationship between individual phytochrome species in the control of lateral root emergence. I was therefore interested to see the effect of reducing the total active phytochrome pool on this response. In this experiment I grew Columbia (Col) (expressing *DR5::GUS*) and Landsberg *erecta* (Ler) seedlings on vertical plates at a photon fluence rate of 70 μmol m^{-2} s^{-1} and a R:FR ratio of 0.12. In keeping with previous experiments, under low R:FR light my seedlings displayed elongated hypocotyls and petioles, and smaller cotyledons when compared to seedlings grown under high R:FR ratio light conditions (Figure 2.2b,c). Col seedlings produced fewer lateral roots than Ler seedlings (Figure 2.2a), however, both ecotypes produced fewer lateral roots when exposed to low R:FR ratio light. The response to low R:FR ratio light was moderate but significant for both ecotypes.

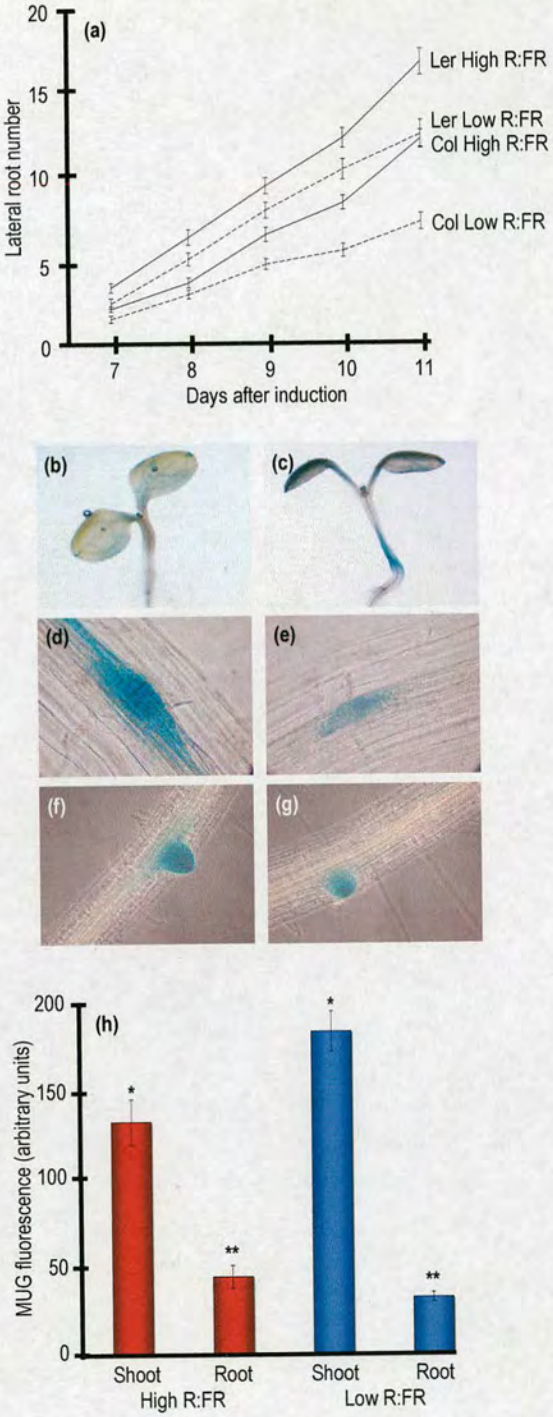


Figure 2.2: Lateral root production and *DR5::GUS* expression patterns in seedlings grown under low and high R:FR ratio light.
Lateral root emergence in Ler and Col seedlings expressing *DR5::GUS* grown under high or low R:FR ratio light (a); *DR5::GUS* expression in 5 day old seedlings (b-e), and 10 day old seedlings (f,g); grown in either high (b,d,f) or low (c,e,g) R:FR. *DR5::GUS* expression, quantified by fluorometric assay, in shoots and roots of 5 day old seedling. In all experiments seedlings were grown in 16h photoperiods at 18°C. Standard error bars are shown. GUS expression in shoot and root tissues was significantly different under high and low R:FR ratio light (*$p<0.025$, $T=-2.37$, $df=26$; **$p<0.022$, $T=2.42$, $df=27$).

Recent work has provided evidence that lateral root emergence rate in young seedlings is controlled by an auxin pulse from the shoot (Casimiro et al., 2001; Bhalerao et al., 2002). I was interested in establishing whether phytochrome controlled lateral emergence by moderating the early shoot-root auxin pulse, or by an independent mechanism. To explore these possibilities I assessed auxin levels and distribution in Col seedlings expressing the synthetic auxin responsive promoter *DR5::GUS* construct (Ulmasov et al., 1997) under high and low R:FR ratio light. Seedling shoots and roots were assayed for *GUS* activity at day 5 in these two experimental conditions. Seedlings exposed to low R:FR ratio treatment had significantly higher shoot *DR5::GUS* expression than those in high R:FR ratio light conditions. This was due to increased *GUS* activity in the cotyledons and the lower portion of the hypocotyl (Figure 2.2c). *DR5::GUS* expression was lower in root tissue compared to shoot tissue in high R:FR grown seedlings, and this was difference was more marked in seedlings grown under low R:FR ratio conditions. Thus, it appears that for seedlings grown in low R:FR ratio light, auxin is redistributed to the shoot, at the detriment of the root. These data suggest that reduced lateral root production in response to low R:FR occurs at least in part by phytochrome-mediated redistribution of auxin.

phyB mutants have less auxin at the root tip than wild type seedlings

I have shown that reducing the R:FR ratio causes a redistribution of auxin in favour of the shoot, thereby causing a reduction in the emergence of lateral roots. As my analysis of *phyB* seedlings showed that they had reduced lateral root production, I was interested to know whether *phyB* seedlings also had reduced auxin levels. A technique that enables quantification of very small differences in auxin levels in root tips has recently been developed (Ljung et al., 2005). In collaboration with Karin Ljung and Goran Sandberg (Swedish University of Sciences) I used this technique to quantify auxin levels in root tips of

Ler (WT) and *phyB* seedlings. The results are shown in figure 2.3a. IAA levels were assayed in root tips from 7 day old seedlings. *phyB* seedlings had less auxin at the root tip than wild type seedlings ($p<0.07$, $T=2.13$ $df=4$). These data therefore support my hypothesis that reduced production of lateral roots in *phyB* mutants is due to a reduction of auxin in the root. Furthermore, these data substantiate my analysis of *DR5::GUS* levels in low R:FR ratio light, indicating that in these conditions, when the active phytochrome pool is severely depleted, root tissues are depleted in auxin.

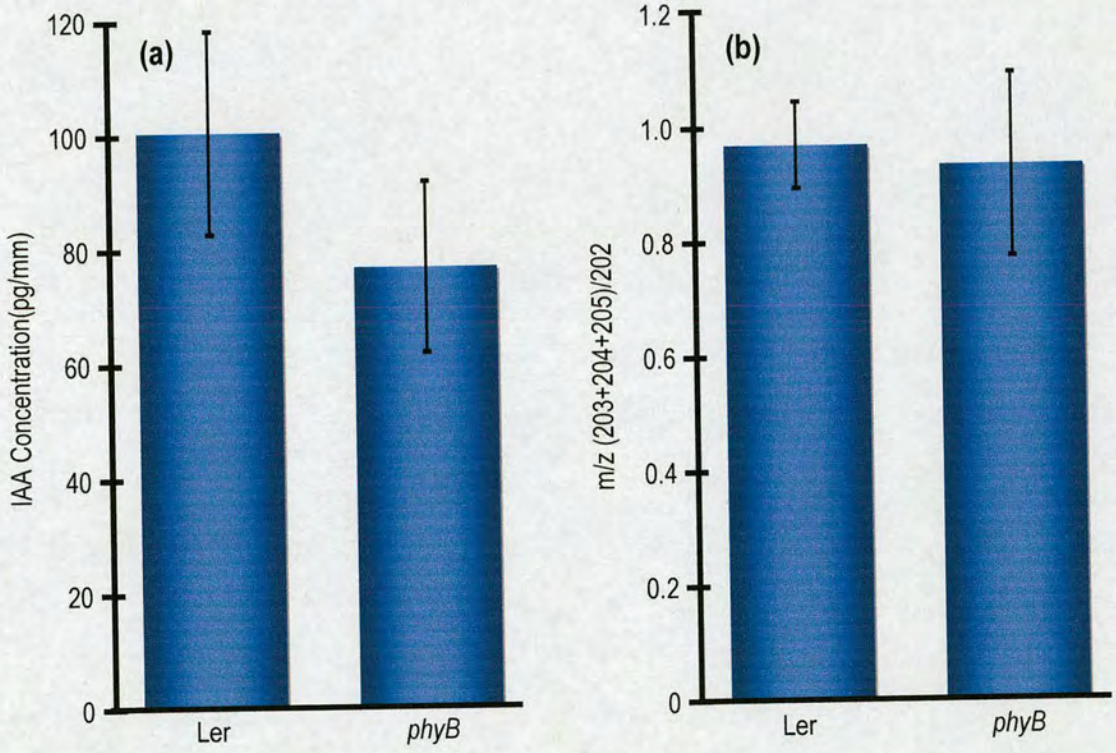


Figure 2.3 IAA levels and biosynthesis in Ler (WT) and *phyB* mutants
IAA concentration in 2mm sections of root tip (a); Auxin biosynthesis in root tips - seedlings were transferred to 30% deuterated water medium for 24 hours before root tips were excised. Deuterium incorporation was measured using GC-HR-MS. Samples were measured in triplicates, and corrections were made for background and natural isotope abundance (b). Seedlings were grown in 16h photoperiods at 18°C for 7 days. Each sample contained pooled plant material from 50 root tips. Standard deviations are shown.

I was curious to understand the extent to which reduced root auxin was due to a reduction of auxin biosynthesis in the root. To this end, I incubated seedlings in medium containing deuterated water for 24 hours before extracting root tips. The incorporation of deuterium into IAA allows quantification of the rate of auxin biosynthesis (Ljung et al., 2005). Figure 2.3b shows that auxin biosynthesis is comparable in root tips of both Ler (WT) and *phyB* seedlings. This indicates that reduced levels of auxin in the root tips of *phyB* seedlings is not caused by a reduction of auxin biosynthesis in this region. These data therefore further support my theory that reduced lateral root production in *phyB* seedlings, and those exposed to low R:FR light is caused by a reduction in auxin from the shoot, and not from reduced synthesis of auxin within the root itself.

DISCUSSION

The phytochromes act collectively to control lateral root production rate

It is well known that phytochromes interact to exert fine control over shoot development in response to light (Casal et al., 2003). When grown under continuous red light, phyB is the major regulator of hypocotyl elongation and cotyledon expansion, whilst the other phytochromes mainly act redundantly with phyB to control these responses (Franklin et al., 2003; Franklin and Whitelam, 2004). I used mutants lacking individual, two, or multiple phytochrome species to assess the impact of phytochrome on lateral root emergence. Both *phyA* and *phyE* mutant seedlings produced fewer lateral roots than the wild type at any given time. However, the most striking phenotype was exhibited by the *phyB* mutant, which produced 30% fewer lateral roots than the wild type during the experimental timeframe. These data suggest a major role for phyB and lesser roles for phyA and phyE as positive regulators of lateral root emergence. In contrast, the *phyD* mutant produced more lateral roots than the wild type during the experimental period, suggesting that phyD antagonises the action of phyB, phyA and phyE as a negative regulator of this response. To investigate the genetic relationship between phyB and the other phytochromes in the control of lateral root production we examined mutants null for phyA, phyD or phyE in addition to phyB. The *phyAphyB* mutant exhibited a phenotype that was intermediate to *phyA* and *phyB*, suggesting a genetic interaction between these two pathways. The similarity of *phyBphyD* to *phyB* and of *phyBphyE* to *phyE* indicated that *phyB* was epistatic to *phyD* and *phyE* epistatic to *phyB* for this response. Collectively my results demonstrate complex genetic interplay between the phytochromes in the control of lateral root growth. Furthermore, the gradient of each lateral root emergence time course, and therefore the rate of lateral root emergence was similar between phytochrome null mutants. Thus it appears that the affect of phytochromes on lateral root emergence is to moderate the timing of the emergence of initial lateral roots, and not by controlling the rate of emergence.

My data, along with previous work demonstrates that young *hy5* seedlings produce more lateral roots than wild type plants (Oyama et al., 1997; Cluis et al., 2004). Thus, it appears that in early seedling development phyB and HY5 have opposing roles in this response. This contrasts with their functions in controlling hypocotyl inhibition in similar aged seedlings, where phyB and HY5 both act as negative regulators (Koornneef *et al.*, 1980; Ang and Deng, 1994). In agreement with earlier work, we have also shown that the rate at which *hy5* produces lateral roots falls off markedly after day 9. These data suggest that either HY5 and the phytochromes act independently to moderate lateral root growth, or that the relationship between HY5 and phytochromes in this response is quite complex.

Phytochromes control lateral root production by altering acropetal auxin transport

Lateral root production is highly dependent upon auxin transport. In early seedling development auxin is synthesised in the shoot then transported through the vasculature and by polar auxin transport toward the root tip, stimulating lateral root growth (Bhalerao et al., 2002; Marchant et al., 2002; Friml et al., 2003; Ljung et al., 2005). As the phytochrome mutants were affected in the timing of the emergence of lateral roots, we were interested in whether the phytochromes regulated this response via manipulation of the auxin pulse. To address this question we examined seedlings expressing the *DR5::GUS* construct under low R:FR ratio light, which depletes active phytochrome (Pfr) levels. When grown under low *vs* high R:FR ratio light Col *DR5::GUS* and Ler seedlings both exhibited elongated hypocotyls and reduced lateral root production. For both accessions low R:FR ratio light did not have a dramatic effect on the root when compared to the shoot elongation response. This is likely to be a reflection of the complex phytochrome interactions that control lateral root emergence. Alternatively, we may not have achieved the Pfr/Ptot threshold required to generate a more pronounced root effect. However, we observed notable changes in the spatial *DR5::GUS*

expression in seedlings grown under low R:FR ratio light. In these seedlings *DR5::GUS* expression was enhanced in the shoot and reduced in the root when compared to the control seedlings. Thus, physiological changes in the seedling induced by low R:FR ratio light are accompanied by an alteration of the distribution of auxin within the seedling, with auxin accumulating in the shoot at the apparent expense of the root. These data are consistent with analysis of IAA levels, which showed that *phyB* mutants had less IAA in the root tip than wild type seedlings. Thus it seems that removal of active phytochrome, either by application of supplementary FR light, or by genetic removal, results in a reduction of auxin in the root. Auxin biosynthesis assays showed that the difference in IAA levels was not due to differences in the rate of auxin synthesis in the root tips of wild type or *phyB* seedlings. As the root tip has been identified as the major source of auxin biosynthesis in the root (Ljung et al., 2005), these data suggest that phytochrome regulates lateral root emergence by adjusting the levels of auxin derived from shoot tissues.

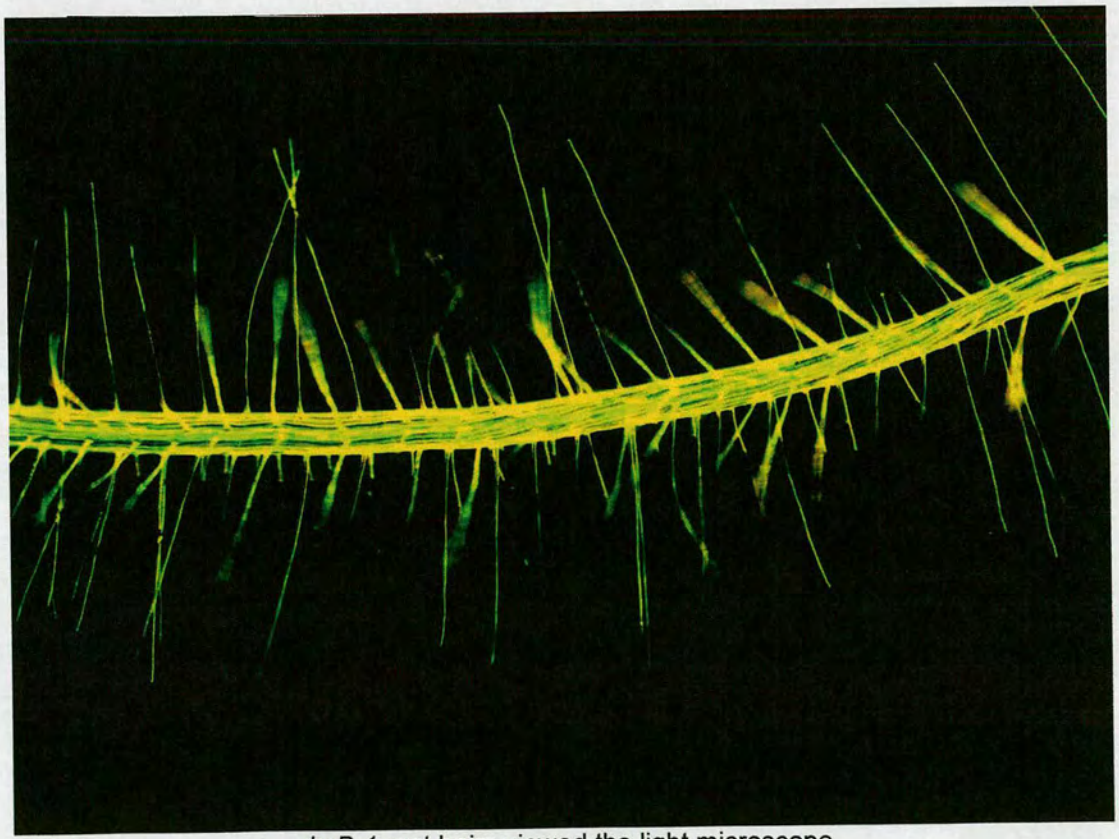
My work is consistent with earlier work that reported a reduced lateral root phenotype in plants overexpressing the phytochrome-regulated homeobox gene *ATHB2* (Carabelli et al., 1993; Steindler et al., 1999; Franklin et al., 2003). This phenotype could be rescued by topical IAA application, suggesting that the *ATHB2* OX phenotype may result from deficiencies in auxin levels (Steindler *et al.*, 1999). These data contributed to a model proposed by Morelli and Ruberti (2000), where low R:FR ratio (shade) light triggers a redistribution of auxin laterally in the shoot, with a consequential reduction in the amount of auxin reaching the root. My data provide some support for such a model, and indicate that ATHB2 would be a downstream effecter in such a model as *ATHB2* levels correlate with phenotype severity (Steindler *et al.*, 1999).

These findings provide a mechanism for co-ordination of shoot and root development in response to the external light environment. At present we do not know how the phytochromes may be acting to control auxin transport, however, recent studies may provide some leads. Microarray data suggests that expression of the auxin efflux effectors *PIN3* and *PIN7* are phytochrome regulated, whilst aspects of the *pin3* mutant phenotype have been shown to be light specific (Friml *et al.*, 2002; Devlin *et al.*, 2003). Therefore, phytochrome may control auxin transport by altering the levels and/or the cellular location of PIN proteins. Alternatively, phytochrome may influence auxin transport through the vasculature. AUX1 has been shown to control the uploading and unloading of auxin to and from the phloem (Marchant *et al.*, 2002). Thus, phytochrome may participate in this process through the regulation of *AUX1* or *AUX1*-like genes. Indeed microarray data also suggests phytochrome regulation of an *AUX1*-like gene.

It is perhaps intuitive that shoot-root communication is essential to synchronise plant growth and development. I have shown that light, a potent regulator of shoot development, has a role in this process, acting through phytochromes. This work provides further evidence for the high levels of integration of the phytochrome and auxin signalling pathways, and provides a novel developmental system to further unravel these interactions.

Chapter 3

Localisation of Phytochromes in Roots, and their Roles in Root Hair Elongation



phyB-1 root hairs viewed the light microscope

CHAPTER 3

THE LOCALISATION OF PHYTOCHROMES IN ROOTS, AND THEIR ROLES IN ROOT HAIR ELONGATION

INTRODUCTION

The *Arabidopsis* family of 5 phytochromes (phyA – phyE) absorb light in the red (R) and far-red (FR) regions of the electromagnetic spectrum. The levels and timing of expression of individual phytochromes are subject to differential control by light and the circadian oscillator (Somers and Quail, 1995; Toth *et al.*, 2001). Thus, the phytochrome pool is dynamic, and acutely responsive to the changing light environment.

Light is absorbed by the tetrapyrrole chromophore, located at the N terminus of the phytochrome apoprotein. This triggers conformational changes throughout the protein moiety, resulting in isomerisation. The ability of the phytochrome molecule to convert between the two isomeric forms gives phytochrome its unique biological activity, as each isomer has a different absorption spectrum – one form absorbing red light (Pr), and the other, far-red light (Pfr). Only the Pfr form is thought to have biological activity (For review see Kevei and Nagy, 2003). This property has enabled experimental manipulation of active phytochrome levels (Whitelam et al., 1998; Franklin and Whitelam, 2004). By providing white light grown seedlings with varying amounts of supplementary far-red light, or end-of-day far-red light treatments, total seedling Pfr can be adjusted dramatically.

Recent studies have revealed that activation of phytochrome is accompanied by changes in the cellular location of the molecule (see Nagatani, 2004). Upon activation by light the

phytochrome molecule undergoes a conformational change that exposes nuclear localisation signals in the PAS domain, and facilitates its nuclear translocation (Chen *et al.*, 2005). This is thought to be important for phytochrome activity, indeed, red light induced nuclear localisation of phyB::GFP was shown to be far-red reversible (Kircher *et al.*, 1999). Several studies have revealed that in the nucleus phytochrome molecules aggregate in subnuclear foci (speckles), whilst speckling intensity has been shown to correlate with severity of response (Kircher *et al.*, 2002; Chen *et al.*, 2003). The precise function of the subnuclear speckling is not yet known, though it has been proposed as the site where phytochrome regulates down-stream signalling events such as protein degradation and transcription. Several lines of evidence are supportive of this view. Phytochrome has been shown to co-localise to subnuclear speckles with CRY2 and with the transcriptional regulator PIF3 (Mas *et al.*, 2000; Bauer *et al.*, 2004). Furthermore, COP1 E3 ligase recruits HY5 to subnuclear foci for degradation by the nucleosome, and COP1 has been shown to be essential for phytochrome-mediated destruction of PIF3 (Ang *et al.*, 1998; Hardtke *et al.*, 2000; Bauer *et al.*, 2004). It is not yet known whether phytochrome interacts with COP1 in subnuclear foci to control these events. Recent work has demonstrated that speckle formation is not essential for all phyB responses, as biological activity has been demonstrated for phyB N-terminal dimers that localise to the nucleus, but do not form speckles, and for diffuse phyB::GFP nuclear staining which occurs at low fluence rates of light (Chen *et al.*, 2003; Matsushita *et al.*, 2003).

The mechanism of phytochrome action outlined above is, however, unlikely to be clear-cut. Following illumination with R light, a high proportion of phytochrome remains in the cytoplasm and in the Pr form indicating that Pr phytochrome may have some role. Although some reports suggest a role for Pr phytochrome (Smith, 1981; Liscum and Hangarter, 1993), extensive research supports the view that *Arabidopsis* phytochromes act only in the Pfr form

(Whitelam et al., 1998). Cyanobacterial phytochromes are however known to act in the Pr form (Karniol and Vierstra, 2003), with autophosphorylation and aspartate phosphotransferase activities of *Synechocystis* phytochrome, CPH1, being specific to the Pr form (Yeh *et al.*, 1997). Plant phytochrome is believed to have evolved from CPH1-like molecules (Montgomery and Lagarias, 2002), and it appears that over time the activities of plant phytochrome have switched from Pr to Pfr.

The localisation of phytochromes and the biological significance of nuclear translocation have been significantly studied in the shoot. Little attention has been directed at the distribution of phytochromes in the root, or their functions. However, there is evidence for phytochrome activity in the root. Roles have been identified for phytochromes in the control of phototropism in roots (Ruppel *et al.*, 2001; Correll *et al.*, 2003; Kiss *et al.*, 2003b). Both primary and lateral roots exhibit positive phototropism in response to red light, and that this response is lost in the primary roots of *phyA* and *phyB* seedlings. PhyD and phyE were also shown to be involved in these responses, but their roles were only minor. Studies using transgenic plants expressing *PHYp::GUS* or *PHYp::LUC* have provided evidence for phytochrome expression in roots (Somers and Quail, 1995; Goosey *et al.*, 1997; Hall *et al.*, 2001; Toth *et al.*, 2001). Thus, roots clearly have the ability to respond to directional light, though this response is largely overridden by the stronger gravitropic response. However, as phytochrome has also been implicated in root gravitropism this provides a mechanism for interplay between these two pathways (Correll *et al.*, 2003).

The role for phytochromes in roots does not appear to be confined to the tropic responses. Early work by Reed and co-workers (1993) demonstrated a role for phyB in the control of root hair elongation. More recently, phytochromes A, B and D have been shown to control red light-mediated elongation of the primary root (Correll and Kiss, 2005). The *hy5* mutant,

known to be defective in phytochrome signalling, also has longer root hairs than wild type (Oyama *et al.*, 1997), further suggesting the involvement of light signalling pathways in root hair elongation.

I was therefore interested to know whether phytochromes were present in roots in my experimental conditions and experimental time frame, and to observe their spatial dynamics within cells. In the previous chapter I showed that phytochromes from the shoot are able to moderate root development. Furthermore, Kiss *et al.*, (2001; 2003b) and Reed *et al.*, (1993) have implicated phytochromes in the control of specific root phenotypes. I was therefore interested to ascertain the extent to which phytochromes control root development.

RESULTS

Phytochromes are expressed in roots and form nuclear speckles in response to light

Phytochrome expression in roots has been reported previously, however, it was unclear to me if and how the expression patterns changed under our experimental conditions, or over the timeframe of my experiments (Somers and Quail, 1995; Goosey *et al.*, 1997; Hall *et al.*, 2001; Toth *et al.*, 2001). I was particularly interested in assessing expression patterns in lateral roots. Transgenic seedlings expressing *PHYA-*, *PHYB-*, *PHYC-*, *PHYD-* or *PHYE-promoter::LUC* constructs were examined in plants grown under 16 hour photoperiods for 7 or 10 days. Seedlings were sprayed with 5mM luciferin and bioluminescence patterns were analysed in the root through *in vivo* imaging. Figure 3.1a shows *PHYA-E::LUC* expression in 10 day old seedlings. The location and distribution of root phytochrome expression was similar in seedlings grown for 7 or 10 days, though expression appeared to be enhanced in the older seedlings (data not shown). At 10 days *PHYA, PHYB, PHYD and PHYE::LUC* activity was observed throughout the entire root, and with the exception of *PHYB::LUC*, high bioluminescence was observed at the root tips of both primary and lateral roots. Interestingly, analysis of *PHYA::LUC PHYD::LUC* and *PHYE::LUC* revealed relatively high levels of bioluminescence throughout the root. Furthermore, *PHYD::LUC* appeared to be highly expressed through the elongation zone of the primary root. To ascertain if this was an artefact caused by light piping I excised the root tip and re-examined the proximal region for bioluminescence. The insert in Figure 3.1a shows that similar levels of *PHYD::LUC* expression are maintained after excision, suggesting that *PHYD* is indeed highly expressed in the elongation zone. The apparent high levels of *PHY::LUC* expression in root tips observed by myself and others may reflect the increased density of cells in this area, rather than a relatively high cellular expression (Hall *et al.*, 2001; Toth *et al.*, 2001). Our experiments do not distinguish between these two possibilities.

In Arabidopsis and tobacco shoots, the cellular location of the phytochrome molecule reflects the activity of the molecule. Light triggers phytochrome translocation to the nucleus, an event that is important for phytochrome activity (Nagatani, 2004). As phytochrome is expressed in roots I wanted to determine whether individual phytochromes exhibited the same light-responsive cellular dynamics in root and shoot cells. To this end, I analysed seedlings expressing PHYA-E::GFP fusion proteins, under the control of the 35S promoter, and PHYB under the control of its native promoter. In agreement with previous studies, I observed cytosolic PHYB::GFP expression in dark grown root epidermal cells (Figure 3.1b-d) (Yamaguchi *et al.*, 1999). Furthermore, as for shoot epidermal cells, I observed diffuse nuclear staining in seedlings grown in red light at 0.1 μmol m^{-2} s^{-1} (~1%Pfr), and nuclear speckling in seedlings grown at 1.0 μmol m^{-2} s^{-1} (~16%Pfr) (Chen *et al.*, 2003). I noted the formation of PHYB::GFP subnuclear foci in a variety of cell types, for example, root hairs, epidermal and the underlying cortical cells (shown in Figure 3.1e,f). I observed identical patterns of localisation using both 35S:PHYB:GFP fusions, and PHYB:GFP expressed under a native *PHYB* promoter. I also observed far-red light-mediated nuclear localisation and speckling of PHYA::GFP, and red light regulation of these events for PHYC::GFP, PHYD::GFP and PHYE::GFP (Figure 3.1g-j). My data demonstrate that the phytochromes are expressed in roots and, furthermore, exhibit similar light-regulated intracellular dynamics in roots as they do in shoots.

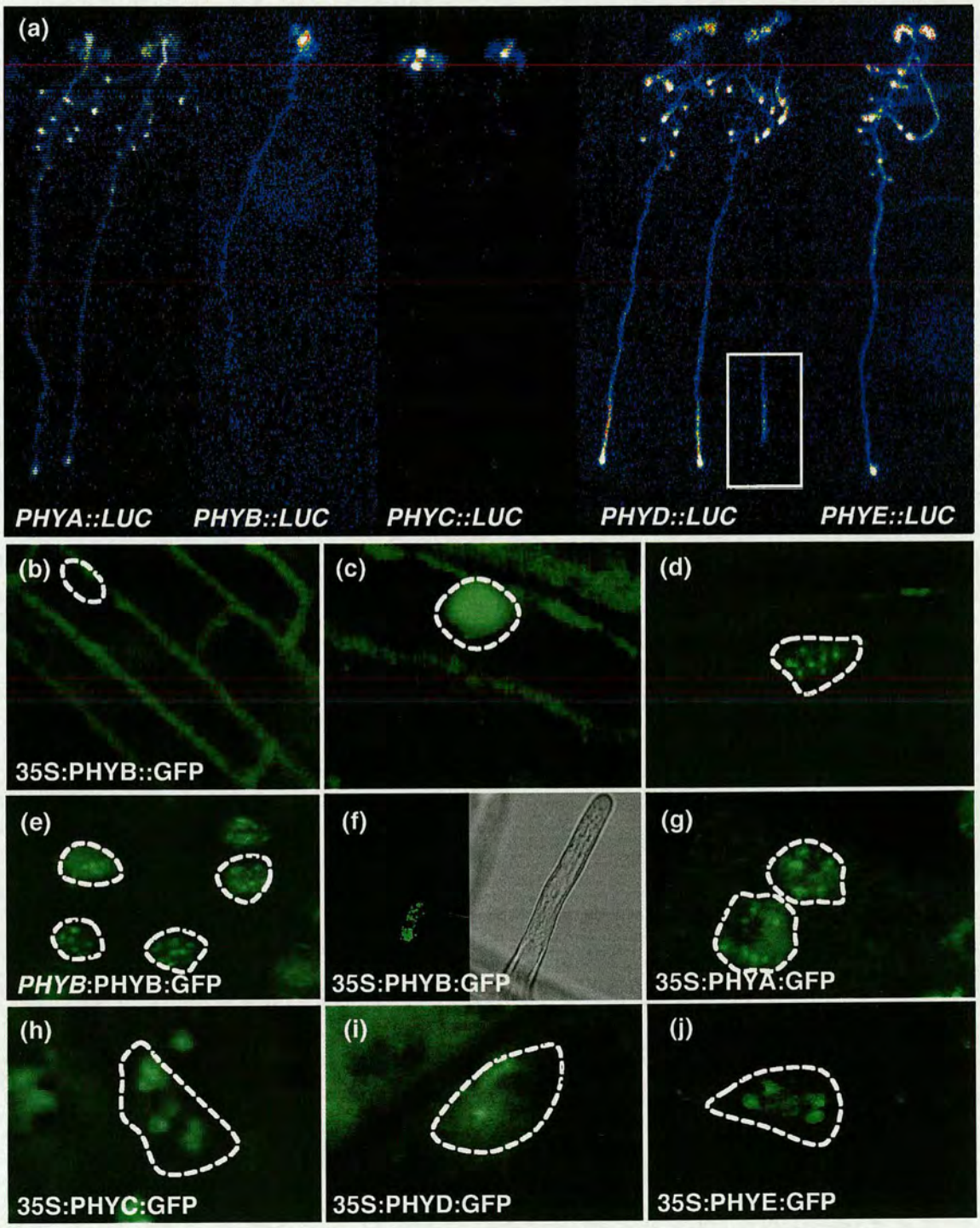


Figure 3.1: *PHY::LUC* expression and *35S*:PHY:GFP cellular localisation in roots. *PHYA-E::LUC* spatial expression patterns are shown for 10 day old seedlings (a). Insert shows *PHYD::LUC* expression following excision of root tip. Cellular location of PHYA-E::GFP in roots of 7 day old seedlings (b-j). phyB::GFP localization patterns in root epidermal cells of seedlings grown in darkness (b), 0.1 μmol m^{-2} s^{-1} (c), and 1 μmol m^{-2} s^{-1} (d) of red light, corresponding to 0%, 1% and 16% Pfr, respectively. Subnuclear speckling of *PHYB*:PHYB::GFP in root epidermal cells (e), *35S*:PHYB:GFP in a root hair cell (f); subnuclear speckling of PHYA, PHYC-E::GFP in root epidermal cells (g-j) in response to far-red (phyA) or red (phyC-E) light.

Phytochrome null mutants have altered root hair phenotypes

Previously, Reed and co-workers (1993) provided evidence for a role of phyB in the control of root hair length, whilst (Oyama *et al.*, 1997) demonstrated a similar role for the phytochrome signalling component, *HY5*. I was interested in determining if other phytochromes also influenced this process. Furthermore, I was keen to find out whether individual phytochrome members had similar genetic relationships in the control of root hair elongation and lateral root emergence. This would provide clues to whether phytochrome was operating via the same mechanism to control lateral root emergence and root hair elongation. In these experiments we measured fully elongated root hairs in the mature zone of the root in wild type and phytochrome null mutants at 7 days (Figure 3.2). In line with previous work, in my experiments *phyB* null mutants had longer root hairs than the wild type, as did the second control, *hy5* (Reed *et al.*, 1993; Oyama *et al.*, 1997) (Figure 3.2a). In contrast to the *phyB* mutant, root hair length in the *phyA, phyD* and *phyE* mutants was similar to the wild type. These data support previous work that suggests a role for phyB and HY5 in the negative regulation of root hair elongation and provide the first evidence that phyA, phyD and phyE are not involved in this process.

To explore possible redundant effects, I analysed seedlings null for *phyA* or for *phyD* and/or *phyE* in addition to *phyB* (Figure 3.2b,c). Seedlings that were deficient in phyA in addition to phyB had a wild type root hair length. These data suggest that for control of root hair length, the *phyA* mutation is epistatic to *phyB*. Thus, phyA appears to be required for the elongated root hair phenotype of *phyB* mutants. In contrast, root hairs in the *phyBphyD* mutant were a similar length to the elongated *phyB* null, suggesting that phyD does not have a significant role in regulating hair length in these genetic backgrounds. However, *phyBphyE* and *phyBphyDphyE* root hairs were wild type in length. Thus, as for *phyA*, *phyE* appears to be epistatic to *phyB* for this response. These results suggest that for the control of root hair

elongation, phyB requires phyE. Furthermore, they also demonstrate that *phyA* and *phyD* exhibit different genetic relationships with *phyB* in the control of lateral root emergence and root hair elongation.

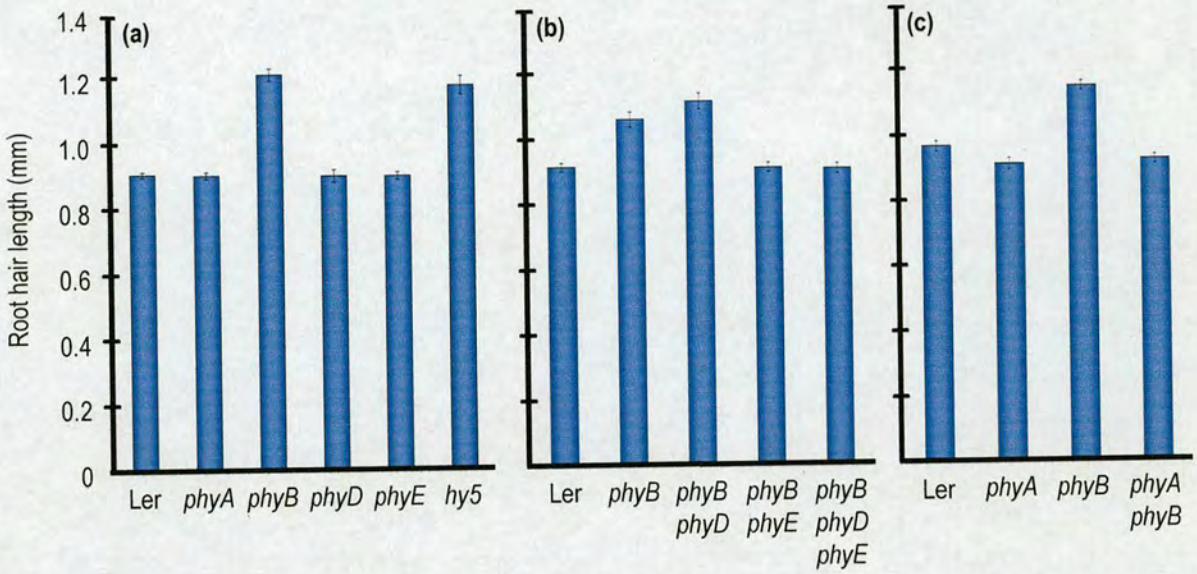


Figure 3.2. Root hair phenotypes of phytochrome deficient mutants.
Root hair length of Ler (WT), phyA, phyB, phyD, phyE and hy5 (a); Ler (WT), phyA, phyB, and phyAphyB (b); and Ler (WT), phyB, phyBphyD, phyBphyE and phyBphyDphyE. Mean root hair length was calculated for over 100 fully elongated root hairs in at least 30 individual seedlings. Standard error bars are shown.

Application of low R:FR ratio has marginal effects on root hair elongation

I have shown that phytochromes are able to modify root hair growth. I was interested to observe the effects of inactivating a large proportion of the phytochrome pool. To this end, I applied FR light to seedlings and measured the lengths of fully elongated root hairs after 7 days. Application of supplementary FR light switches a large proportion of the phytochrome pool to its inactive form. In shoots this treatment produces similar effects to those observed in mutants lacking *phyB*.

Figure 3.3 shows that root hairs on seedlings exposed to low R:FR ratio light were only a tenth longer than those in high R:FR light, whereas *phyB* mutants can have root hairs one

third longer than the length of those of a wild type plant. At the R:FR ratios used we would expect the phyB Pfr pool to be strongly reduced, yet the root hair phenotype was only one third of the effect of the *phyB* mutation. In contrast to the *phyB* null mutant, exposure to low R:FR ratio light has a significantly smaller effect on root hair elongation growth, and may reflect the complex nature of the interactions between phytochrome species controlling this phenotype.

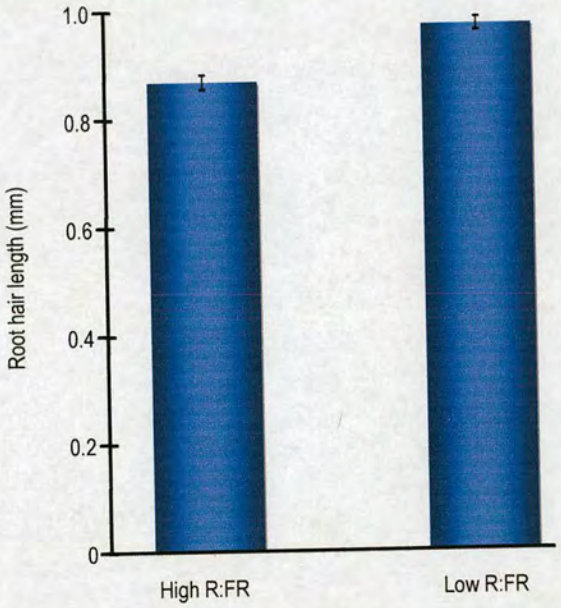


Figure 3.3: The effect of low R:FR ratio on root hair length in wild type (Ler) seedlings. Root hairs of seedlings exposed to low R:FR light treatment. Mean root hair length was calculated for over 100 fully elongated root hairs in at least 30 seedlings. Bars show standard error.

The C7g chromophore-deficient phyB overexpresser line is not defective in lateral root production or root hair elongation

I wanted to test whether phytochrome was able to moderate root hair development independently of this molecule's role in light perception. To this end, I measured the lengths of fully elongated root hairs in ABO seedlings, a *PHYB* overexpresser line, and C7g, a line overexpressing similar levels of a mutated phyB that is unable to incorporate the

photosensory chromophore. In both lines, similar quantities of the wild type or mutated PHYB protein are overexpressed (Hennig et al., 2001). Both of these lines are in the Nossen (No-0) background, and were compared to Ler and *phyB* seedlings as controls. If the chromophore was not required for this aspect of root development, then ABO and C7g seedlings should have similar root hair length. Results are shown in figure 3.4a.

In line with my previous analysis, and earlier published work (Reed *et al.*, 1993), *phyB* seedlings had longer root hairs than wild type (Ler). The ABO *PHY*B overexpresser line had shorter root hairs than wild type (No-0), supporting a role for phyB as a negative regulator of root hair length (Reed *et al.*, 1993; Salisbury *et al.*, In preparation). Root hairs of the C7g chromophore deficient overexpresser line were also shorter than wild type (No-0), though were not as short as hairs of the ABO line. These results therefore support my hypothesis that phytochrome may be able to regulate root hair elongation independently of its photoperception activity.

In the previous chapter I demonstrated that phytochromes are involved in the regulation of lateral root emergence. I was interested to know whether this phenotype required the chromophore. I established the numbers of lateral roots for ABO and C7g seedlings, along with their wild type (No-0) between 7 and 11 days following induction with white light. Ler and *phyB* seedlings were used as controls, as I have previously characterised their lateral root emergence (Salisbury et al., In preparation). Results are shown in Figure 3.4b.

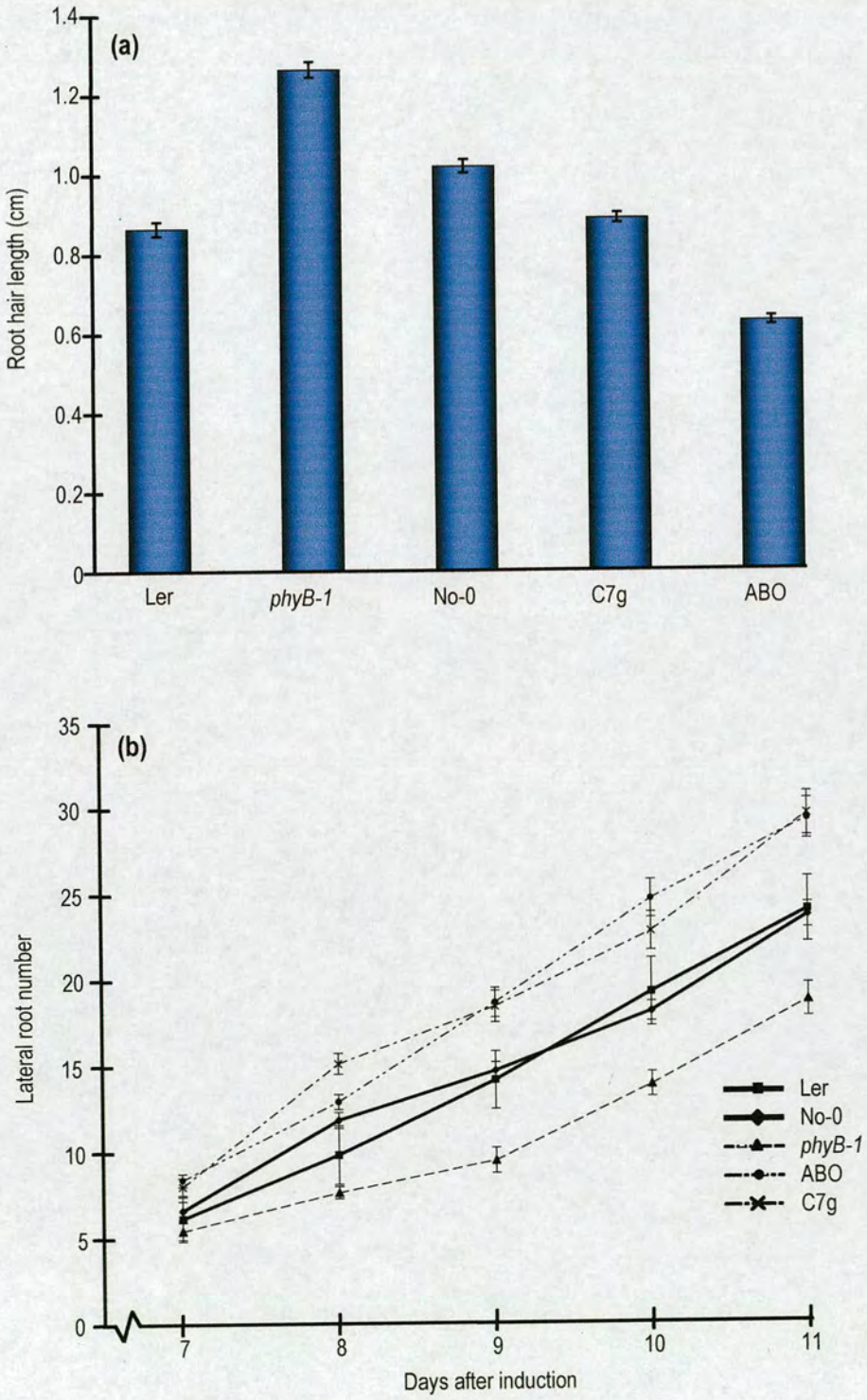


Figure 3.4: Analysis of the C7g chromophore deficient phyB overexpresser line and controls.
a) Length of fully elongated root hairs in the mature zone of the root after 7 days of light. b) Number of lateral roots emerged between 7 and 11 days following transfer to light. Seedlings were grown at 18°C in continuous white light, fluence 150 mol μmol m^{-2} s^{-1}. Data are representative of replicate experiments of at least 20 seedlings each. Standard error bars are shown.

Ler and No-0 wild type seedlings produced lateral roots at similar rates. In line with my previous work, *phyB* mutants produced fewer lateral roots at any given time point than wild type (Ler) (Salisbury et al., In preparation; Chapter 2), implicating this phytochrome as a positive regulator of lateral root emergence. In accordance with this, the ABO phyB overexpresser line produced more lateral roots than wild type (No-0). Interestingly, the C7g chromophore deficient seedlings produced similar numbers of lateral roots to ABO. This indicates that regulation of lateral root emergence, and at least to some extent root hair elongation, is not dependent on the photoactivity of the phytochrome molecule.

Phytochrome null mutants have root phenotypes when grown in darkness

To establish whether phytochrome could act in a light independent manner in roots, I also took an alternative, complementary approach. I assessed the impact of phytochrome loss in seedlings grown in complete darkness. I have previously reported that phyA and phyB are positive regulators of lateral root emergence (Salisbury et al., In preparation), and wanted to see if this phenotype is light regulated. I assessed the numbers of emerged lateral roots in seedlings 10 days post germination (Figure 3.5a). When grown in the dark, the *phyAphyB* double mutant had fewer lateral roots than the wild type, and the *phyB* monogenic mutant had even fewer lateral roots. Thus, the *phyB* and *phyAphyB* mutant phenotypes exhibited identical trends when compared to wild type, when grown in either light or dark conditions. This lends support to the hypothesis that phytochrome has a light-independent action in the root. Previous reports (Oyama *et al.*, 1997; Cluis *et al.*, 2004; Salisbury *et al.*, In preparation), and data presented in this thesis (Figure 2.1) have shown that *hy5* seedlings grown in the light produce more or similar numbers of lateral roots to wild type. In the dark, *hy5* produced only half the laterals of the wild type. These data indicated that the negative

regulation of lateral root emergence by HY5 requires light, and suggest a positive role for HY5 in this response in the dark.

As earlier work had suggested a role for phyB in the regulation of primary root length in dark-grown seedlings (Reed *et al.*, 1993), I was interested in assessing my seedlings in this respect. These results are shown in Figure 3.5b. In agreement with the findings of Reed and co-workers, my dark grown *phyB* mutants had shorter primary roots when compared to wild type seedlings. Furthermore, removal of *phyA* in addition to *phyB* resulted in a shorter root, suggesting that these phytochromes act independently to control this phenotype. Previous work showed that the primary root of light grown *hy5* mutants was shorter than wild type (Oyama *et al.*, 1997). Interestingly, the primary root length of *hy5* was not different to the wild type when grown in the dark, indicating that HY5 control of primary root length is light dependent.

Hypocotyl lengths all of the genotypes tested were not significantly different from wild type seedlings (Figure 3.5c). This is interesting and may indicate that phytochrome actions are different in shoots and roots, and that root phytochromes are able to act independently of light to regulate root development.

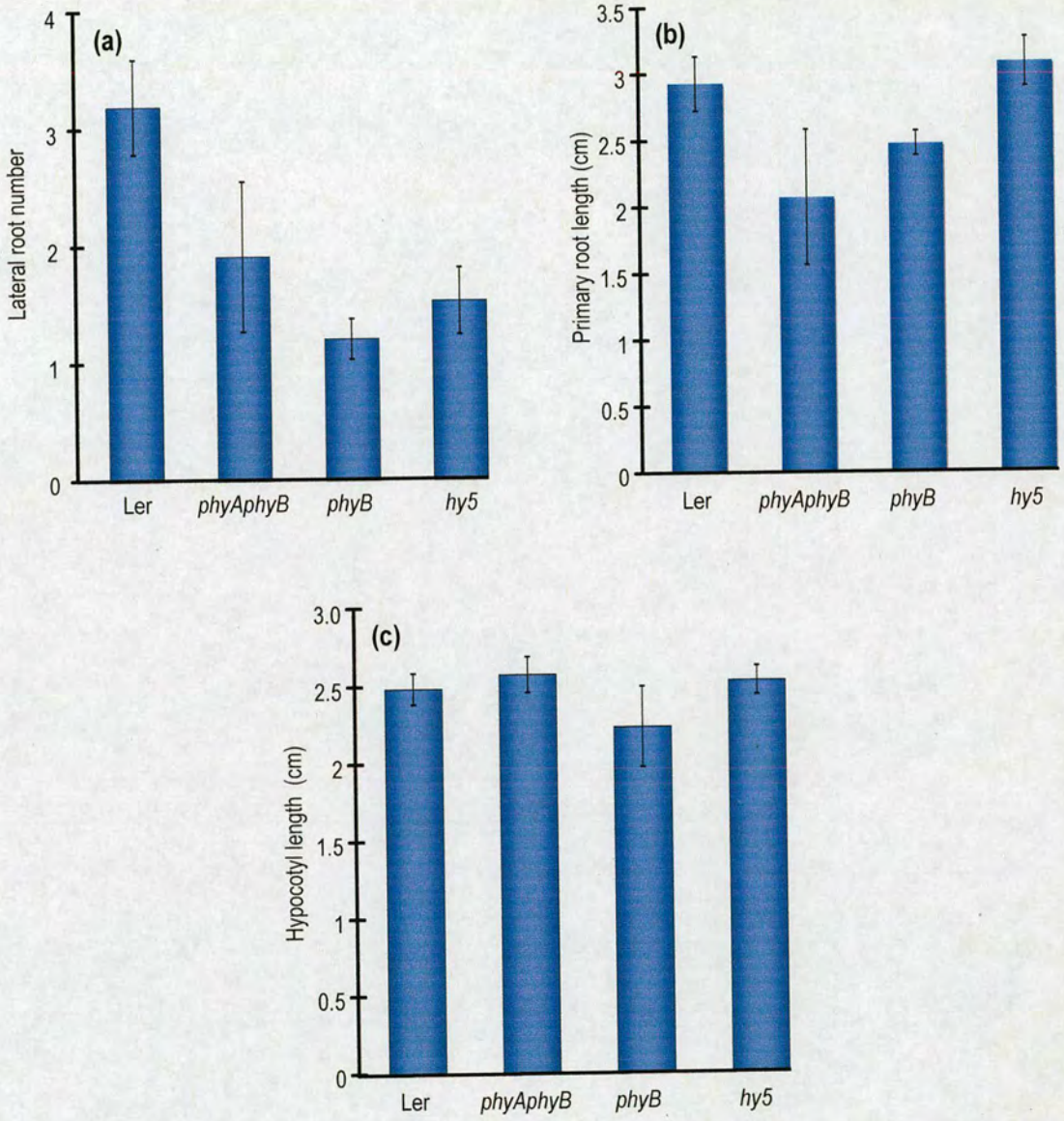


Figure 3.5: Phenotypes of seedlings grown in complete darkness.
Number of emerged lateral roots (a), length of the primary root (b), and hypocotyl length (c) of seedlings were grown in complete darkness for 10 days at 18°C. Data represent a preliminary experiment with samples of at least 10 seedlings per genotype. Standard error bars are shown.

DISCUSSION

Phytochromes are expressed and are functional in root cells

Recent work using lines expressing *PHY::LUC* has shown that the phytochromes are expressed in the root, albeit to much lower levels than the shoot (Hall *et al.*, 2001; Toth *et al.*, 2001). Previous studies have also demonstrated changes in *PHYB, PHYD* and *PHYE::GUS* expression patterns during seedling development (Somers and Quail, 1995; Goosey *et al.*, 1997). Thus, I was keen to establish whether phytochrome expression patterns changed under my growth conditions and experimental timeframe. At 7 and 10 days *PHYA, PHYD, and PHYE::LUC* exhibited similar patterns, with expression foci in the primary and lateral root tips. *PHYB* and *PHYC::LUC* expression was very low in our transgenic seedlings. I am uncertain if this is an accurate reflection of expression status of these genes, particularly as we and others have demonstrated a prominent role for phyB in the regulation of root-specific responses (Kiss *et al.*, 2003a; 2003b; Correll and Kiss, 2005). It is possible that the *PHYB:LUC* transgene has been incorporated into a region of the genome with low transcription activity, or may lack important 5' sequences that promote gene expression. It would be interesting to compare these data with gene expression analysed by RNA *in situ* hybridization or qPCR, and with expression patterns of transgenes containing longer transcripts.

A number of groups have provided insights into phytochrome function by connecting the dynamic cellular properties of phytochrome with its function. Activation by light induces conformational change to the active Pfr form and translocation to the nucleus, followed by the formation of diffuse staining and/or subnuclear foci or speckles (Sakamoto and Nagatani, 1996; Kircher *et al.*, 1999; Yamaguchi *et al.*, 1999; Gil *et al.*, 2000; Kim *et al.*, 2000; Chen *et al.*, 2003). These characteristics hold true for each of the phytochrome species (Kircher *et al.*, 2002). Several studies have shown a strong correlation between subnuclear foci

formation and phytochrome function. Mutant forms of phyA and phyB, known to have either no or reduced physiological activity, do not aggregate in nuclear foci (Kircher *et al.*, 2002; Chen *et al.*, 2003). Furthermore, nuclear focus size has been shown to correlate with strength of the physiological response (Chen *et al.*, 2003). Recent work also suggests a role for nuclear phyB that is not associated with subnuclear speckles. N-terminal phyB which translocates to the nucleus, but remains dispersed is more active than full length phyB (Matsushita *et al.*, 2003). Indeed, under low fluence rate red light conditions, which generate diffuse phyB nuclear staining, plants are still light responsive (Chen *et al.*, 2003). As these studies have been conducted predominantly in shoot cells, I wanted to ascertain whether the phytochromes possessed the same characteristics in root cells. My work has shown that this is indeed the case. I observed light regulated cytosolic to nuclear translocation and the formation of subnuclear speckles for PHYA-E::GFP in root epidermal cells. Furthermore, I also observed diffuse nuclear PHYB::GFP in root epidermal cells of seedlings grown under low fluence rate red light. These data suggest that phytochrome can act within the root to control local growth responses. Indeed, recent work has shown that red light inhibits primary root elongation and this is partly mediated through photoreceptor action in the shoot and partly through photoreceptor action in the root (Correll *et al.*, 2003). These workers also showed that primary root elongation was regulated by multiple phytochromes. Thus, it appears that phytochrome may indeed act within the root to regulate growth, in addition to moderating phototropism (Kiss *et al.*, 2002; 2003b)

In a previous chapter I demonstrated that phytochromes regulate lateral root production, at least partly by altering auxin transport (chapter 2). I was interested to establish whether this was the main mechanism via which phytochrome exerted its effects on root development. Furthermore, I was curious to understand whether phytochromes that I had shown to be present in root cells could act locally to control aspects of root development. To provide

insights into this question we assessed the roles of individual phytochromes in a second response, the control of root hair length, and tested their genetic relationships. Previous work had already established a role for phyB in the negative regulation of root hair elongation (Reed *et al.*, 1993). Likewise, HY5 has also been shown to negatively regulate this process via an *IRE*-dependent pathway (INCOMPLETE ROOT HAIR ELONGATION), suggesting similar roles for phyB and HY5 in regulation of root hair growth (Oyama *et al.*, 1997, 2002). Consistent with these previous reports, in my experiments *phyB* and *hy5* had long root hair phenotypes (Reed *et al.*, 1993; Oyama *et al.*, 1997, 2002). However, *phyA*, *phyD* and *phyE* mutants were essentially wild type for this response. Removal of *phyD* in addition to *phyB* did not have a significant effect on the *phyB* phenotype indicating that phyD does not regulate root hair elongation in the genotypes tested. However, analysis of the mutants lacking phyA or phyE in addition to phyB revealed essential roles for these phytochromes in control of root hair growth. *phyA* and *phyE* were both epistatic to *phyB* suggesting that phyA and phyE are both required for phyB control of root hair elongation. These data contrast with my analysis of lateral root production, in which monogenic mutant analysis established roles for phyA, phyB, phyD and phyE. Furthermore, I have also demonstrated a genetic interaction between *phyB* and *phyA* that leads to an intermediate phenotype, and *phyB* epistasis with *phyD*. Thus, my data demonstrate different contributions for individual phytochromes and different genetic relationships between phytochrome species for control of lateral root production and root hair elongation. This suggests that these two phytochrome-controlled responses are mediated via different mechanisms. Support for this notion also comes from my *DR5::GUS* analysis. Auxin has been shown to promote root hair elongation, yet low R:FR ratio light reduces auxin levels in the root and enhances root hair elongation. Therefore, it appears that the phytochromes cannot act solely by moderating the shoot-root auxin pulse to regulate root development. This provides the possibility that

phytochrome is controlling root development via an additional shoot-derived signal, or that phytochrome is operating within the root system itself to control aspects of development.

The control of root hair elongation may have environmental significance. Root hairs are of significant value to a growing plant, increasing surface area by up to 75%, thereby enhancing the capability of the plant to absorb water and nutrients, and by providing anchorage (Gilroy and Jones, 2000; Bailey et al., 2002). Coupling root hair elongation to light signalling could allow nutrient and water absorption to be tailored to the growth of the plant - *phyB* seedlings, for example, have elongated shoots compared to wild type, and may require additional water and nutrients. It would therefore be advantageous for these seedlings to have the additional anchorage that elongated root hairs provide. Alternatively, phytochrome control of root hair elongation may represent a role for this molecule outside of light signalling.

Phytochromes may be able to act in the Pr form to regulate root phenotypes

The central dogma of phytochrome biology is the ability of phytochrome to photoconvert between two isomers, one biologically active, and the other not. Indeed, a great body of evidence has been amassed to suggest that this is the case. However, there have been a few studies proposing a role for the inert Pr form (Smith, 1981; Liscum and Hangarter, 1993; Reed et al., 1994; Kim et al., 1998; Correll and Kiss, 2005), though these have largely been ignored by the wider research community.

My analysis of root hair elongation phenotypes of wild type seedlings grown under FR enriched conditions, under which most of the phytochrome pool would be expected to be in the inactive Pr form led me to speculate that Pr phytochrome may be able to regulate root hair elongation. Root hairs of seedlings grown under FR enriched conditions are not significantly longer than those under white light. This could reflect the complex interactions

of phytochromes in controlling root hair elongation, but may also indicate that phytochrome control of root hair elongation is not dependent on the conversion of phytochrome to the Pfr form. To investigate this possibility further, I looked at root hair elongation in transgenic seedlings expressing a chromophoreless form of phyB.

The Pr form of phytochrome can control root hair length and lateral root emergence

The C7g line of transgenic seedlings expresses a phytochrome holoprotein that carries a C357S mutation. This phytochrome is unable to incorporate the light sensing chromophore, and therefore does not undergo light regulated conformation changes. I assessed lateral root emergence and root hair length in these seedlings. I used seedlings that overexpress phyB to similar levels (ABO) as a control, along with wild type seedlings (No-0) (Hennig et al., 2001). If the Pr form of phytochrome were active, then C7g and ABO lines would display similar phenotypes. My results showed that, as expected, the ABO lines produced shorter root hairs (Figure 3.4a), and lateral roots were formed more rapidly than in wild type seedlings (Figure 3.4b). These results are opposite to those seen in *phyB* mutants (Reed *et al.*, 1993; Salisbury *et al.*, In preparation). Furthermore, the C7g lateral root phenotype was remarkably similar to that of the ABO transgenic line, and the root hair phenotype intermediate between those of the wild type and ABO plants, providing support for my hypothesis that Pr can be active in roots.

These results corroborate my theory that light is not required for a subset of phytochrome-mediated responses. However, they must be interpreted with caution. C7g seedlings contain both modified phyB without chromophore (overexpressed C357S phyB) and native, intact phyB. Thus it is possible that the expressed C357S phyB could cause a dominant negative effect whereby modified phyB competes with native phyB for interaction pathways, thereby blocking phyB signalling. However, as the C7g root phenotype resembled ABO, rather than

the *phyB* null, my data suggest that the overexpressed C357S phyB is able to overcome any deficiencies in phyB signalling caused by possible dominant negative effects. It is also possible that C357S phyB could affect signalling in phytochrome pathways other than phyB, though this would represent a function of Pr phytochrome.

Some phytochrome mediated root phenotypes are maintained in the dark

Phytochrome is converted to the active Pfr form in response to light absorption. Therefore, an alternative approach to investigating whether Pr phytochrome could moderate root phenotypes would be to examine the phenotype of phytochrome deficient seedlings grown in darkness. To this end I assessed lateral root emergence, and primary root length, phenotypes already known to be phytochrome mediated and regulated by C357S phyB (see above). If phytochrome could act in the Pr form to control these phenotypes then I would expect a similar pattern of results to those already reported for these seedlings when grown in the light. In the light, *phyB* seedlings produce fewer lateral roots (Salisbury et al., In preparation) and have a shorter primary root length (Reed *et al.*, 1993). In my experiments dark-grown seedlings of the *phyAphyB* mutant produced fewer lateral roots than wild type, and *phyB* seedlings produced even fewer lateral roots than *phyAphyB* (Figure 2.2a,b). This is qualitatively the same phenotype as seen in light grown seedlings (Salisbury et al., In preparation). Primary roots of *phyB* seedlings were shorter than wild type, and the phenotype of *phAphyB* was even more severe. These results provide support for my proposal that phytochrome regulates a subset of responses that do not require light. Furthermore, as hypocotyl length was not different between genotypes, these results provide evidence that shoot and root phytochromes may function differently to regulate growth.

Maintenance of phytochrome dependent phenotypes in darkness has recently been observed by Correll and Kiss (2005). Analysis of root elongation in phytochrome null mutants

implicated phyA and phyB in this phenotype in the light. Furthermore, roots of *phyA*, *phyB*, and *phyAphyB* mutants failed to elongate as wild type in darkness. These data therefore provide further support for my hypothesis that phytochromes are able to act in the dark, and therefore outside of light perception to moderate root phenotypes.

Phytochrome signalling in darkness has also been reported by Kim *et al.*, (1998). These workers investigated the *shy2-1D* mutation. SHY2 is an Aux-IAA protein (IAA3), and therefore a suppresser of auxin signalling. When grown in darkness *shy2-1D* seedlings maintain photoresponsive phenotypes, such as a short hypocotyl, and cotyledon expansion. These plants continue to undergo development in the dark, forming foliar leaves and floral organs. Furthermore, removal of phytochrome in a *shy2-1D* background reduced the photomorphogenic responses, indicating that phytochromes are able to operate in the dark in *shy2-1D*. Phytochrome deficient responses were also observed in the *shy2-1Dhy2* double mutant. *hy2* mutants are deficient in chromophore biosynthesis. Reduced photomorphogenic phenotypes in the double mutant therefore implicate the phytochrome holoprotein, and suggests a role for Pr phytochrome in regulating photoresponsive phenotypes in darkness.

HY5 is bZIP transcription factor that is known to be a signalling component lying downstream of both phytochrome and cryptochrome photoreceptors in light grown seedlings. It is unknown whether this relationship is maintained in dark grown tissues. Importantly, Cluis and co-workers (2004) have also identified HY5 as an integrator of light and hormone signalling pathways. Given these roles for HY5 I was interested in establishing if it was a candidate signalling component downstream of phytochrome in light-independent signalling. Thus, *hy5* mutants were included in my analysis of dark grown seedlings. *hy5* mutants have previously been reported to have more lateral roots than wild

type seedlings when grown in the light (Oyama *et al.*, 1997; Cluis *et al.*, 2004; Salisbury *et al.*, In preparation), and also have shorter primary roots (Oyama *et al.*, 1997). My results show that, when grown in the dark, *hy5* seedlings produced fewer lateral roots than wild type (Figure 3.5a). It would therefore appear that, HY5-mediated negative control of lateral root emergence is dependent on light. Furthermore, the similarity of the *hy5* phenotype to that of *phyAphyB* may indicate that HY5 may act in the same, or a complementary pathway to these two photoreceptors in the dark.

HY5 is also known to affect primary root length as *hy5* seedlings have shorter roots than wild type seedlings when grown in the light (Oyama *et al.*, 1997). My results show that this phenotype is not maintained in the dark, with *hy5* having a primary root of equivalent length to wild type (Figure 3.5b). These data indicate that HY5 mediated control of primary root length is dependent on light. In contrast, dark-grown *phyB* and *phyAphyB* mutants both have shorter roots than the wild type. These data suggest that phyA and phyB act independently of light to control this phenotype and that HY5 may not be involved in this response.

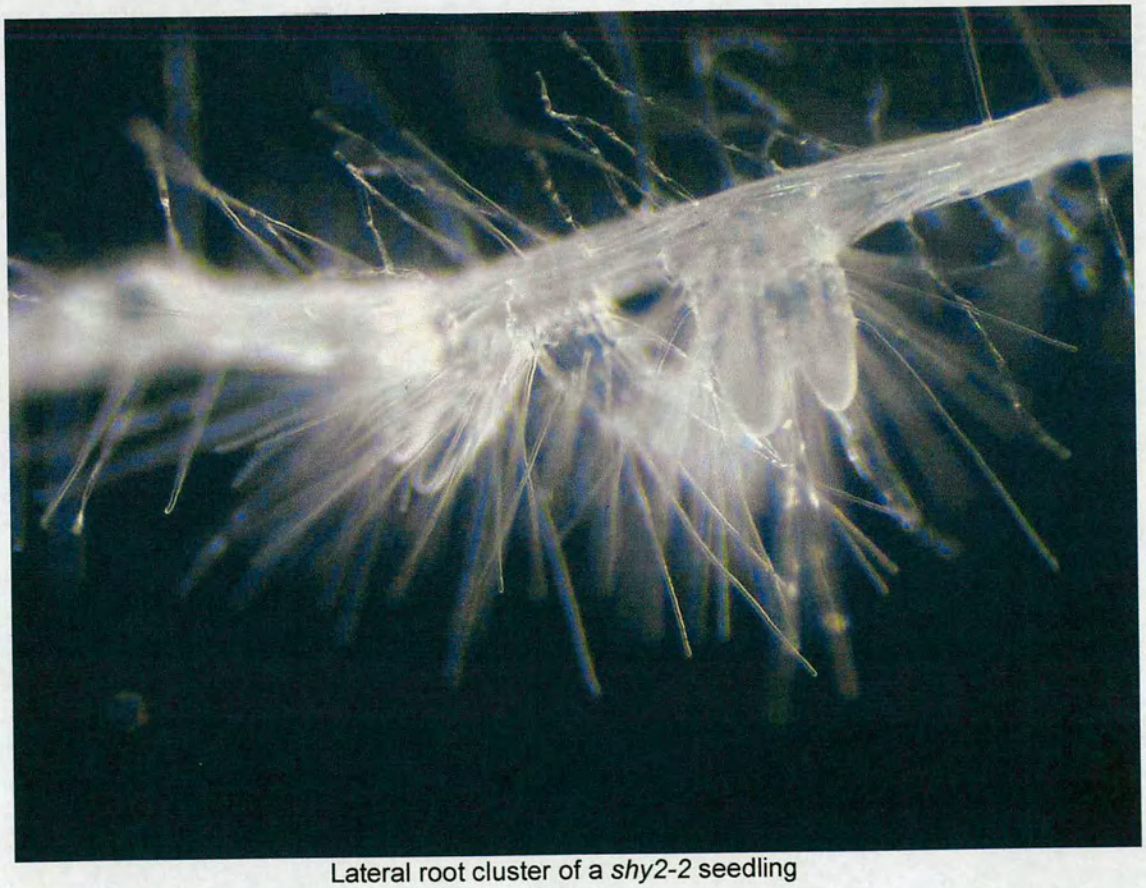
My data from dark grown seedlings lend further support to my idea that light is not required for a subset of phytochrome responses in *Arabidopsis* roots. However, as with results from my studies using the C7g transgenic seedlings, caution must be exercised in interpreting these results. Magliano and Casal (2004) have recently shown that pre-germination signals from phytochrome within seeds are able to control hypocotyl elongation. Phytochrome within a seed could be converted from Pr to Pfr thus generating a signal that is stored within the seed, and could subsequently modulate growth during germination. Therefore it remains a possibility that the dark seedling phenotypes that I have observed may therefore be a result

of light perceived by the seed before transfer to dark conditions. However, this would require a prolonged signal to regulate growth up to 10 days post germination.

My data, combined with published evidence therefore provide substantial support for the hypothesis that the inactive, Pr form of phytochrome is able to moderate a range of responses. This work therefore directly challenges the central dogma that phytochrome is only able to act as a light receptor. Though controversial, if I provide conclusive evidence, this will have a major impact on how we view phytochrome signalling.

Chapter 4

SHY2 and phyB Interact Genetically to Regulate Root Hair Elongation



Lateral root cluster of a *shy*2-2 seedling

CHAPTER 4

SHY2 AND PHYB INTERACT GENETICALLY TO REGULATE ROOT HAIR DEVELOPMENT

INTRODUCTION

Root hairs are long tubular outgrowths from specialist epidermal cells. They are of significant importance to plants, making up as much as 75% of the surface area of the root, and therefore act as a major point of interaction between plant and soil (Grierson and Ketelaar, 2004). Root hairs participate in nutrient and water uptake, and are involved in interactions between plants and pathogenic and symbiotic bacteria (including nitrogen fixing bacteria in legumes).

In Arabidopsis, root hair patterning follows a distinct developmental programme. Cells of the epidermis either differentiate into hair forming trichoblasts or non-hair forming atrichoblasts, depending on their position relative to underlying cortical cells. Only cells overlying the anticlinal wall between two adjacent cortical cells become trichoblasts, with cells overlying only one cortical cell becoming atrichoblasts. Consequently, trichoblasts tend to form in non-adjacent files of cells. Analysis of mutants with aberrant epidermal patterning has identified several genes involved in this process, including *GL2, TTG* and *CPC* (*GLABRA* 2, *TRANSPARENT TESTA GLABRA* and *CAPRICE*) (reviewed in Schiefelbein, 2000).

Once cell fate has been determined, root hair formation begins towards the apical end of the root hair cell. Acidification of the cell wall and activation of xyloglucan endotransglycosylase and expansin proteins results in localised thinning and loosening of the cell wall, and formation of a bulge. A localised region within this bulge then grows out via a rapid tip growth to form a root hair. Cell wall components are delivered to this region by cytoplasmic streaming, contained within vesicles derived from the endoplasmic reticulum and Golgi bodies. These secretory vesicles are directed to a tightly controlled region of the root hair tip, allowing localised elongation of the root hair at around 1μm min^{-1}. As tip growth proceeds, the nucleus moves into the growing root hair, moving behind the tip until growth ceases. Once the root hair is fully elongated the nucleus retreats in to the main body of the root hair cell (for review see Dolan, 2001).

Tip growth requires development of a calcium gradient, arising from activation of Ca^{2+} channels at the root hair tip (Bibikova *et al.*, 1999). The growth rate of root hairs correlates to the size of this gradient, and can be inhibited by application of calcium channel blockers. Root hairs of *rhd2* mutants fail to elongate, remaining at the bulge initiation stage. This is because they lack an NADPH-oxidase regulator of calcium channel activity, and consequently are unable to establish a Ca^{2+} gradient (Foreman *et al.*, 2003). The exact function of the calcium gradient remains unclear, but it is involved in controlling the direction of growth. Recent work in pollen shows that the Ca^{2+} gradient is established after growth, and is required for actin disassembly by RIC3 (Gu *et al.*, 2005).

Local increases in Ca^{2+}, using UV-activated caged ionophores, are sufficient to alter the direction of tip growth. When used in conjunction with oryzalin, a drug that depolymerises microtubules, local increases in Ca^{2+} can cause formation of an additional growing tip, and consequently lead to a branched root hair phenotype (Bibikova *et al.*, 1999). Similar

concentrations of oryzalin, without calcium manipulation, result in wavy root hairs, and increasing the concentration results in increasingly branched root hairs. Similar waving and branching phenotypes are also observed in root hairs treated with taxol, a drug that stabilises microtubules (Bibikova *et al.*, 1999). Further indication of the importance of microtubules comes from analysis *mor1* seedlings, which have disrupted microtubule organisation, and in transgenic lines with reduced α-tubulin expression, both of which produce branched root hairs (Bao *et al.*, 2001; Whittington *et al.*, 2001). Microtubules are therefore important for maintaining a single growth point at the root hair tip, and for ensuring that elongation occurs in a straight line.

Whilst disruption of the microtubule cytoskeleton results in the branching and waving of root hairs, the rate of root hair elongation and the width of root hairs are not changed. These aspects of root hair development are regulated by the actin cytoskeleton. In root hairs, thick actin filaments run parallel to the long axis in root hair cells (net axial FB-actin) (Miller *et al.*, 1999), and other bundles of actin run through cytoplasmic strands running through the vacuole. Disruption of actin dynamics using cytochalasin D or latrunculin B causes root hair growth to stop, despite the persistence of cytoplasmic streaming, and often results in root hairs with deformed tips (Baluska *et al.*, 2000). Pulsed application of low levels of either of these drugs is not sufficient to stop growth, and causes in an increase in the diameter of root hairs (Ketelaar *et al.*, 2003). Taken together, these data identify a role for actin in directing polar growth, probably by targeting and releasing Golgi-derived vesicles to the vesicle rich region at the root hair tip, and for limiting the area of growth and maintaining a uniform width along the growing root hair.

Many mutants have been isolated that exhibit similar phenotypes to those observed following cytochalasin or latrunculin treatments. Mutants with disrupted *ACTIN2* (*act2-1*

and *der1*) produce root hairs with varying widths and often initiate multiple hairs from a single bulge site (Gilliland *et al.*, 2002; Ringli *et al.*, 2002), further implicating actin in determination of the area from which tip growth can occur, and in the maintenance of tip growth. The severe root hair phenotype of *act2-1* seedlings can be rescued by *ACT1* and *ACT7* indicating a degree of redundancy between individual actin proteins, and suggesting that the expression pattern of *ACT2* is important for the role of this gene in root hair development. However, although absence of *ACT7* has no effect on root hair development in the presence of *ACT2* (Gilliland *et al.*, 2002; 2003).

The plant hormones auxin and ethylene are also important positive regulators of root hair development. Auxin response mutants *axr2* and *axr3* fail to produce root hairs, and *ctr1* ethylene response mutants produce ectopic root hairs (Wilson *et al.*, 1990; Kieber *et al.*, 1993; Leyser *et al.*, 1996). Exogenous treatment with the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) leads to production of ectopic root hairs (Tanimoto *et al.*, 1995), and conversely, application of the ethylene biosynthesis inhibitor AVG (aminovinylglycine) inhibits root hair production. IAA or ACC treatments are also able to recover the root hair initiation defect of *rhd6* mutants (Masucci and Schiefelbein, 1994). Auxin and ethylene are therefore implicated in root hair initiation. IAA treatment alone does not however induce ectopic hair formation, despite induction of ethylene biosynthesis in roots by auxin (Masucci and Schiefelbein, 1996). Thus, it is the combined action of auxin and ethylene that is required for root hair initiation. This is further supported from the observation that *aux1-7ein2-1* double mutants initiate even fewer root hairs than either single mutant (*ein2-1* mutants are ethylene insensitive) (Pitts *et al.*, 1998). Furthermore, treatment of *ein2-1* roots with auxin transport inhibitors results in a decrease in the number of root hairs, indicating that normal root hair initiation in this mutant requires normal auxin signalling (Rahman *et al.*, 2002).

Auxin and ethylene also regulate root hair elongation. Treatments with the synthetic auxin 2,4D or with ACC result in the development of longer root hairs than untreated controls (Pitts *et al.*, 1998). Reduced ethylene signalling in *etr1* (*ETHYLENE RECEPTOR)* and *ein2* (*ETHYLENE INSENSITIVE)* mutants results in the production of very short root hairs (Pitts *et al.*, 1998). However, these mutants also produce some wild type root hairs, indicating that sufficient residual ethylene response persists in these mutants to allow root hair elongation. This concurs with data indicating that these mutants still respond to exogenous application of ACC (Pitts *et al.*, 1998). Ethylene is therefore a positive regulator of root hair elongation, possibly due to the effects of this hormone on cell wall polymers, through regulation of nucleotide sugar flux (Seifert *et al.*, 2004). Mutants with reduced auxin signalling also exhibit root hair elongation phenotypes. *aux1* seedlings, for example have a short root hair phenotype which may be recovered by exogenous auxin (Rahman *et al.*, 2002). As for root hair initiation, auxin and ethylene interact to regulate root hair elongation. This is illustrated well by analysis of *axr1-12* root hairs, which were shown to be hypersensitive to ACC treatment. Conversely, *ein2-1* root hairs have been shown to be resistant to IAA (Pitts *et al.*, 1998; Rahman *et al.*, 2001).

Gain of function mutants in two Aux/IAA genes produce opposing root hair phenotypes, and have provided insights into the mechanism by which auxin regulates root hair development. *axr3/iaa17* mutants produce fewer and shorter root hairs than wild type, with severe alleles having a hairless phenotype, whereas *shy2-2/iaa3* gain of function seedlings have an increased density of longer root hairs than wild type. Knox *et al.*, (2003) used a heat shock promoter to control expression of these two genes, and found that these Aux/IAA proteins affected different stages of development. *AXR3* blocks root hair initiation and elongation, whereas *SHY2* promotes early initiation and extended duration of root hair elongation.

Double-mutant analysis indicated that *SHY2* and *AXR3* act simultaneously in a dose dependent manner to regulate root hair development, and that regulation of the relative amounts of *AXR3* and *SHY2* is important for the control of root hair development.

I have previously shown that *phyB* mutants have reduced lateral rooting, and have shown that this is at least in part attributable to reduced levels of auxin reaching the root (chapter 1). *phyB* mutants also have longer root hairs than wild type seedlings (Reed *et al.*, 1993; Salisbury *et al.*, In preparation). As auxin is a positive regulator of root hair elongation, reduced levels of auxin in the root of *phyB* seedlings would be expected to result in decreased, and not increased root hair length. I was interested in investigating this discrepancy and understanding the mechanism by which phyB regulates root hair length. To this end, I took a genetic approach and investigated the root hair phenotypes of *shy2-2phyB-1* seedlings. *shy2-2* was chosen as a candidate as *shy2-2* seedlings, like *phyB-1* seedlings exhibit a long root hair phenotype. Also, *shy2-2* has been isolated as a suppressor of the long hypocotyl phenotype of both *phyB* and *hy1* (deficient in chromophore biosynthesis) mutants (Reed *et al.*, 1998) and *SHY2-2* and *PHYB* have been shown to interact in an *in-vitro* pulldown assay (Tian *et al.*, 2003). Thus, *SHY2-2* was a likely candidate for phyB-mediated regulation of root hair elongation.

RESULTS

shy2-2 seedlings, like *phyB-1* seedlings produce long root hairs (Reed *et al.*, 1993; Knox *et al.*, 2003). The *shy2-2* mutation was originally identified as a suppressor of *phyB-1*, and SHY2 has been shown to interact with phyB in an *in-vitro* pull down assay (Tian *et al.*, 2003). I was therefore interested to understand whether SHY2 and phyB acted together or independently to regulate root hair elongation. To this end, I grew seedlings on vertically orientated plates, and compared root hairs of *shy2-2phyB-1* double mutants with those of wild type, *shy2-2* and *phyB-1* single mutant seedlings.

Under my growth conditions, both *phyB-1* and *shy2-2* seedlings produced longer root hairs than wild type (Ler: 0.60mm±0.015, *phyB*: 0.90mm±0.05, *shy2-2*: 0.82mm±0.02). This result is in line with previous reports (Reed *et al.*, 1993; Knox *et al.*, 2003). If phyB and SHY2 acted independently to regulate root hair elongation, then the double mutant would be expected to have longer root hairs than each parental line. However, *shy2-2phyB-1* seedlings have a striking root hair phenotype, producing waved and highly branched root hairs (Figure 4.1e-g) compared to wild type (Figure 4.1a). Wild type root hairs are straight projections from an epidermal cell, and are of a similar width along most of their length, whereas *shy2-2phyB-1* root hairs are wavy and produce many branches from one root hair. As the double mutant phenotype was so strikingly different from either parental line, it is evident that *phyB-1* and *shy2-2* interact genetically to regulate root hair length.

I was interested in establishing the stage in root hair development at which the *shy2-2phyB-1* interaction was important. I looked at root hairs of 7 day old seedlings using scanning electron microscopy (SEM). Micrographs clearly showed that root hairs grew in files of cells that were not adjacent. Only one hair formed per cell, and this hair grew at the end of the cell nearest the root tip. This indicated that *shy2-2phyB-1* seedlings were not defective in

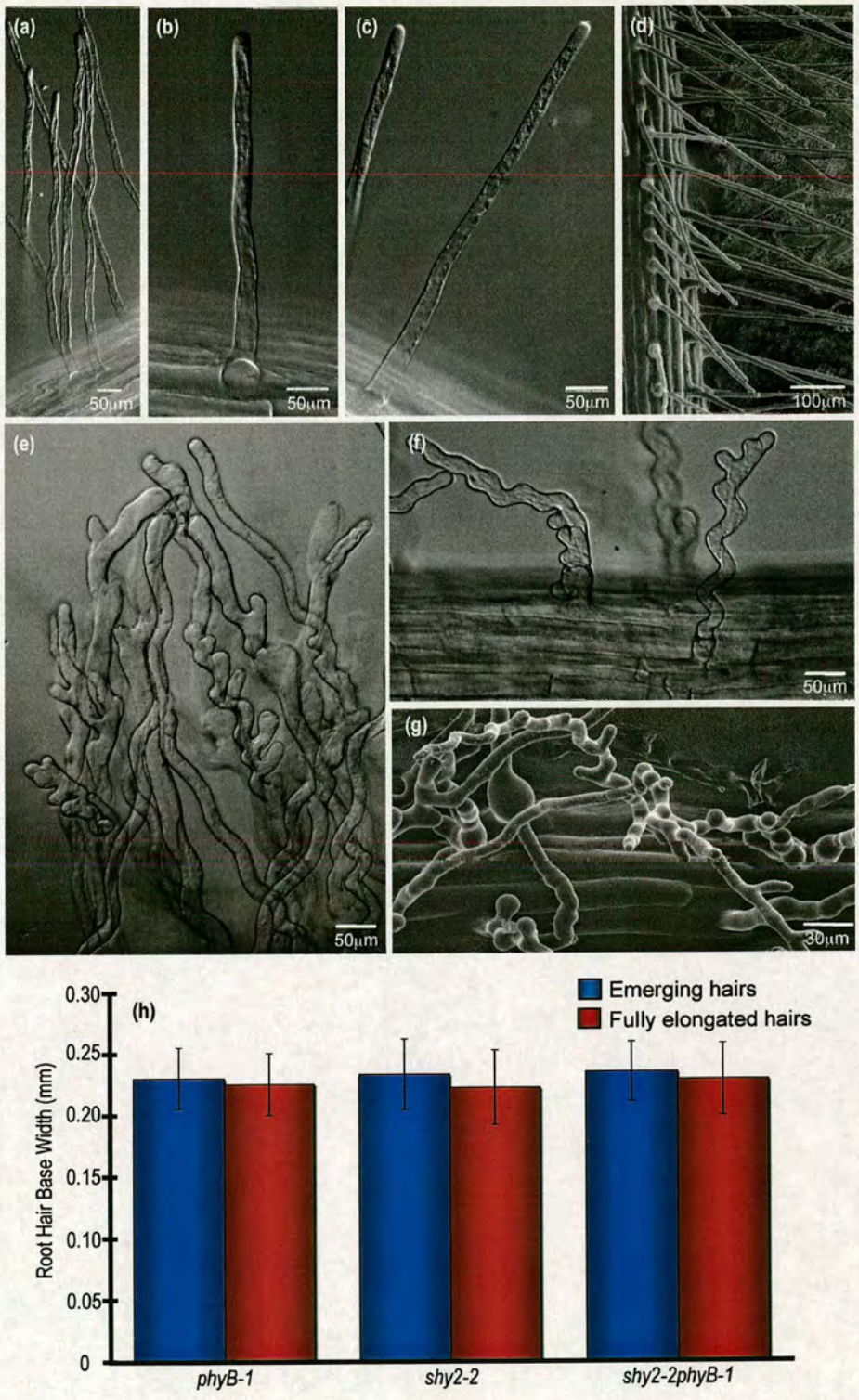


Figure 4.1: Root hair phenotypes of *shy2-2phyB-1* seedlings.
Differential interference phase contrast (DIC) microscopy images of root hairs in wild type (a), *shy2-2* (b), *phyB-1* (c) and *shy2-2phyB-1* (e,f) seedlings. Wild type (d) and *shy2-2phyB-1* (g) root hairs viewed by SEM. Images represent average phenotypes of at least 30 seedlings. Widths of bulge sites of emerging and fully elongated root hairs (h). Data represent mean bulge widths of at least 35 immature and mature hairs. Standard error bars are shown.

bulge site selection, and root hair patterning. To assess the impact of the interaction on bulge site initiation, I measured the widths of the bases of emerging root hairs on 7 day old seedlings, using light micrographs. The results are shown in figure 4.1h. Bulge site width of double mutant root hairs was identical to each parent line, and to the wild type (data not shown). I also measured bulge width in mature hairs, and confirmed that bulge site did not change during root hair development. Thus it appears that in *shy2-2phyB-1* double mutant seedlings early stages of root hair development, including root hair patterning and hair initiation are unaffected.

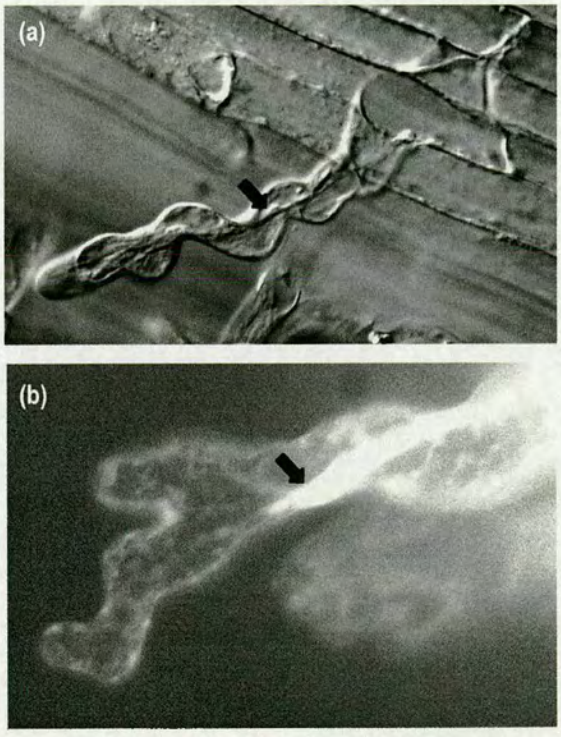


Figure 4.2: Cortical cytoplasm in root hairs of *shy2-2phyB-1* seedlings.
shy2-2phyB-1 root hairs visualized by DIC light microscopy showing a filamentous structure running from base to tip (a). A similar filamentous band glows brightly when stained with fluoroscein diacetate and visualized under UV (b).

Thus it seems that the effect of the interaction between SHY2 and phyB is confined to the elongation phase of root hair development. Root hairs of the double mutant have multiple

branches and are extensively wavy, indicating that the growing root hair is unable to maintain a single point of growth. This phenotype bears a striking resemblance to seedlings treated with the microtubule disrupting drugs and oryzalin and taxol.

Close inspection of *shy2-2phyB-1* root hairs revealed a filamentous structure running from the tips to the base of the root hair. Cytoplasmic streaming in growing hairs appeared to run alongside this structure. A similar feature is seen after immunofluorescent labelling of seedlings treated with microtubule disrupting drugs, so it seems that the genetic interaction between *phyB* and *shy2-2* regulates the microtubule network of root hairs. A similar fluorescent structure is seen in fluoroscein diacetate treated root hairs of *rhd3* mutants (Galway *et al.*, 1997; Bibikova *et al.*, 1999). To establish the composition of this band, I stained root hairs of *shy2-2phyB-1* seedlings in fluoroscein diacetate, mounted them in glycerol and analysed the fluorescence pattern under UV light. The filamentous structure of these root hairs fluoresced brightly under UV (Figure 4.2b), indicating that this band consisted of cortical cytoplasm.

I wanted to understand whether the physical interaction of phyB and shy2-2 observed in *in-vitro* pulldown assays was important *in-vivo*, or was an artefact of the technique. I therefore made a *SHY2*:SHY2:YFP construct to assess colocalisation with phyB, using existing phyB-GFP transgenic lines. Genomic SHY2 DNA containing both the promoter and gene sequences was isolated by PCR. *Sma*I and *Not*I restriction sites were added to the 3' and 5' ends respectively by incorporation into the PCR primers. YFP DNA was isolated from the pEYFP plasmid, and *Not*I and *Sac*II sites were added, again by PCR. PCR products were immediately TA cloned into pCR2.1 TOPO. Once the sequence had been confirmed, the SHY2 fragment was digested using the supplementary restriction sites, and was ligated into pBluescript. The resulting plasmid was then cut and YFP added in frame using *Not*I and

*Sac*II. SHY2YFP was excised from pBluescript using *Sma*I and *Sac*II and cloned into the binary vector pGreenII. *Agrobacterium* mediated transformation was subsequently used to transfer SHY2YFP into *Arabidopsis* plants expressing *PHYB:*PHYB:GFP.

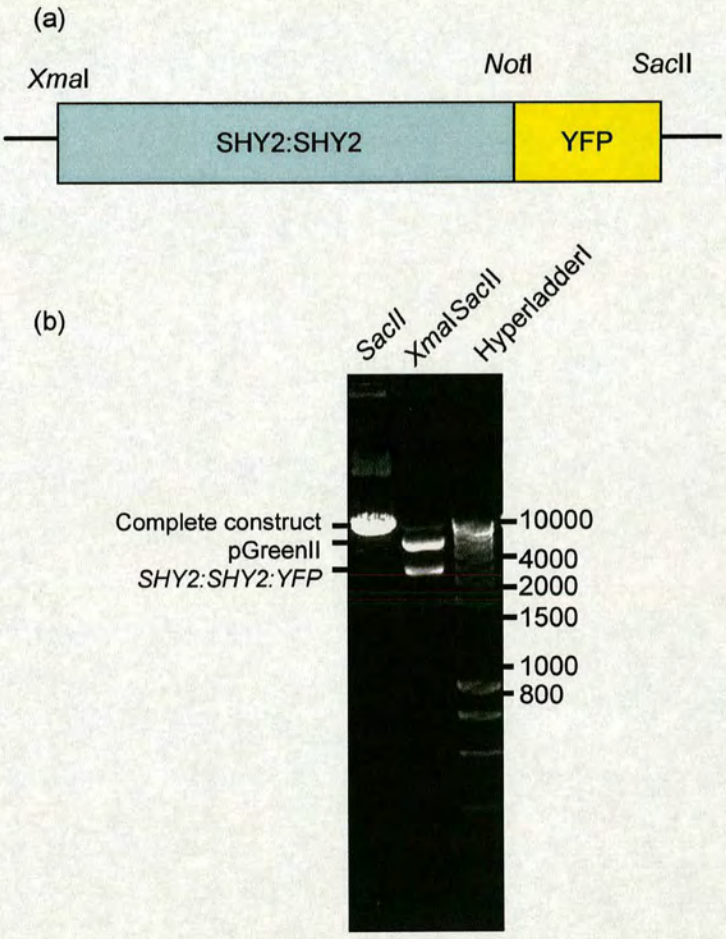


Figure 4.4: Development of the *SHY2*:SHY2:YFP construct
Plasmid map of the completed *SHY2*:SHY2:YFP construct with restriction sites annotated (a). Restriction digests of the complete plasmid (b).

DISCUSSION

In chapter 3 I showed that the phytochromes act together to regulate root hair elongation, with phyB playing a major role. I was interested in understanding how the phytochromes, particularly phyB, integrated with root hair elongation machinery to regulate the length of mature root hairs. Given that auxin plays an important role in root hair elongation, I was particularly interested to know whether the long root hair phenotype of *phyB* mutants resulted from changes in auxin signalling. Gain of function mutants in *SHY2/IAA3,* like *phyB* null mutants, produce longer root hairs than wild type. Furthermore, SHY2/IAA3 and phyB have been shown to interact in an *in-vitro* pulldown assay. I therefore hypothesised that SHY2/IAA3 played a role in phyB regulation of root hair elongation. To test for an interaction between SHY2 and phyB I took a genetic approach and looked at *shy2-2phyB-1* double mutants. If SHY2 and phyB acted independently to regulate root hair elongation then double mutants would have longer root hairs than either parental line. However, *shy2-2phyB-1* root hairs are branched and wavy, indicating that these two genes do indeed function together to regulate root hair elongation. Early stages of root hair development, such as bulge site selection and root hair patterning, and bulge formation appear unaffected in the double mutant, indicating that the interaction specifically affects the elongation phase of root hair development.

Recent work by Bibikova *et al.* (1999) investigated the effect of microtubule disrupting drugs on root hair development. Wild type root hairs treated with oryzalin or taxol are wavy and frequently branched: strikingly similar to *shy2-2phyB-1* root hairs. *shy2-2phyB-1* root hairs have a uniform width, and are not shorter than wild type, indicating that growth is not prematurely arrested. These data therefore indicate that SHY2 and phyB might interact genetically to regulate microtubule dynamics.

Fluoroscein diacetate staining reveals a thick band of cytoplasm running the length of the hair, indicating that the cytoplasm is disrupted in root hairs of the double mutant. A similar band is also observed in hairs with disrupted microtubules (Bibikova *et al.*, 1999), providing further support for a role for *phyB* and *SHY2* in the regulation of microtubule stability. However, this observation must be treated with caution as fluoroscein diacetate is a cytoplasmic stain, and not specific to microtubules. It should also be noted that disrupted cytoplasm could be a feature of distorted root hairs, and not a cause of their phenotype. However, direct comparisons may be drawn between the band observed in *shy2-2phyB-1* root hairs and a similar feature observed in fluoroscein diacetate stained *rhd3* mutants (Galway *et al.*, 1997). *RHD3* encodes a GTP binding protein that is thought to regulate organisation of the actin cytoskeleton (Hu *et al.*, 2003). *RHD3* is required for cell wall biosynthesis, and has been observed to shuttle between the endoplasmic reticulum and Golgi bodies (Zheng *et al.*, 2004). Interestingly root hair tips of *shy2-2phyB-1* are similar to root hairs of seedlings treated with the actin disrupting drugs cytochalasin D and latrunculin B (Baluska *et al.*, 2000), though deformed tips could be a consequence of a late and aborted branching attempt, and therefore be a consequence of altered microtubule dynamics.

Many reports in the literature suggest roles for the phytochromes and auxin as regulators of the microtubule cytoskeleton. Application of continuous red light to etiolated seedlings causes inhibition of growth and results in a longitudinal arrangement of microtubules. Light stable phytochromes are thought to promote stabilisation of microtubules. Red light pulse experiments, however, reveal that light labile phytochrome regulates a transverse, unstable orientation of microtubules. Microtubule orientation is believed to require specific microtubule associated proteins (MAPs), of which several are known to be phytochrome regulated (Leu *et al.*, 1995; Devlin *et al.*, 2003).

Whilst the light stable phytochromes promote a longitudinal arrangement of microtubules, auxin promotes a transverse orientation. Transfer of seedlings from auxin depleted to auxin medium promotes reorientation of cortical microtubules from longitudinal to transverse. This process is anaerobic, indicating that it is a direct response to auxin application, and not a consequence of cell elongation (Takesue and Shibaoka, 1998). Furthermore, auxin depleted seedlings have higher levels of detyrosinylated or stabilized α-tubulin. Fischer and Schopfer (1997) used immunofluorescent techniques to investigate the combined effects of phytochromes and auxin on microtubules during phototropism. They visualized microtubules under different concentrations of IAA, and under red and far-red lights and concluded that auxin and light signals act synergistically to orientate microtubules, with microtubules responding to the sum of the reorientation signals, rather than each signal independently.

Phytochrome and auxin are therefore implicated in the regulation of microtubule orientation and stability. This leads me to propose a model whereby decreased root auxin resulting from the absence of phyB, and increased stabilisation of a negative regulator of auxin signalling would increase the proportion of stabilized microtubules. Consequently, normal root hair development would be impaired, resulting in the observed waving and branching phenotypes. If this model were correct one would anticipate that application of the microtubule destabilizing drug oryzalin to *shy2-2phyB-1* roots would result in a recovery of root hairs to wild type. Furthermore, application of low concentrations of oryzalin to wildtype root hairs should result in longer root hairs, similar to those observed in both *phyB-1* and *shy2-2* single mutants. Analysis of microtubule orientations within disrupted root hairs may also provide important evidence, and crosses of the double mutant to transgenic lines expressing GFP tagged tubulin have already been preformed and await selection.

The model centres on the observation that *phyB* causes reduced auxin to reach the root (chapter 2), and consequently results in an increase in the proportion of microtubules in a transverse alignment. However, reduced root auxin in *phyB* mutants would be expected to result in a short root hair phenotype and not the long root hairs observed. Consequently, while this model may go some way to explaining the genetic interaction of between *phyB* and *SHY2*, it is not sufficient to explain the single mutant phenotypes. Other auxin mutants may provide clues to an alternative mechanism. *aux1* mutants have reduced auxin transport, and in line with the observation that auxin promotes root hair elongation has a short root hair phenotype (Rahman *et al.*, 2002). However, another mutant with reduced auxin sensitivity, *axr1* produces longer root hairs than wild type (Pitts *et al.*, 1998). Whilst this observation may be explained as an alteration of the sensitivities of *axr1* trichoblasts to auxin or environmental factors, it is possible that *AXR1* has a distinct role as a downstream component in auxin mediated root hair elongation. *AXR1* encodes a nuclear localised protein with homology to an E1 ubiquitin ligase and is required for activation of RUB1/NEDD8, a ubiquitin related protein (Leyser *et al.*, 1993; Pozo *et al.*, 1998). RUB1 conjugation (neddylation), targeted by AXR1 may be an important process in root hair elongation. Short root hair phenotypes of *aux1* and other auxin signalling mutants may be due to reduced auxin in trichoblasts, and consequently reduced auxin signalling through *AXR1*. Interestingly, SHY2 stability is increased in *axr1-1*mutants (Tian *et al.*, 2003), indicating that *SHY2* lies downstream of *AXR1* for this response. phyB may act as a positive regulator of *AXR1*, or by directly regulating SHY2 stability. Alternatively the interaction between *SHY2* and phyB may be important at a later stage in the mechanism of root hair elongation. It would therefore be interesting to determine whether phyB is able to interact with AXR1, either physically or genetically, and whether SHY2 is stabilised in *phyB-1* mutants. This model suggests that protein degradation by neddylation could be an important regulator of

root hair elongation by auxin, and determination of the root hair phenotype of seedlings with reduced expression of RUB1 may provide insights.

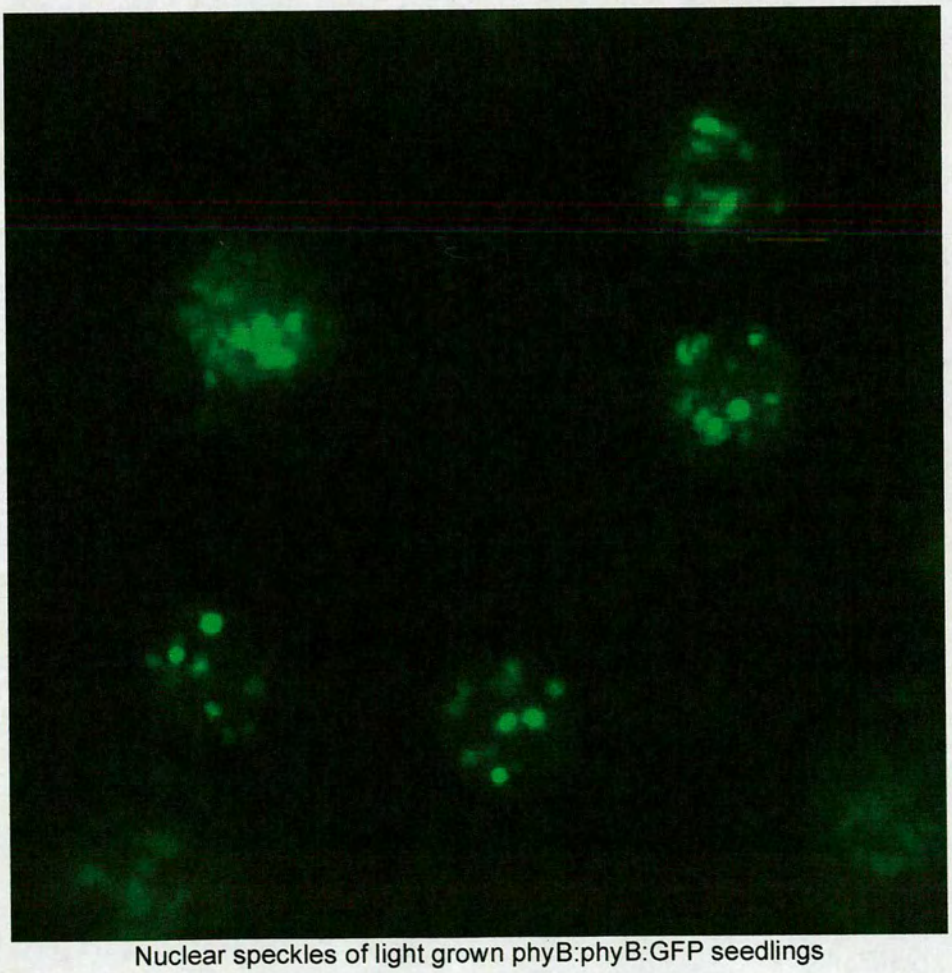
Aux/IAA proteins are important negative regulators of auxin signalling. Auxin promotes degradation of Aux/IAA proteins at SCF^{TIR1}, thereby releasing repression of ARF (AUXIN RESPONSE FACTOR) transcription factors. In Arabidopsis there are 22 members of the ARF family (Remington *et al.*, 2004) and each may be regulated by one or more IAA protein. Specificity of responses is optimised by pairs of interacting IAAs and ARFs, and by transcriptional control of ARF expression (Weijers *et al.*, 2005). SHY2/IAA3 has been shown to regulate gravitropism and auxin induced gene expression in the root through inhibition of NPH4/ARF7 and ARF19 (Weijers *et al.*, 2005). *nph4-arf19* double mutants, like *shy2-2* mutants have severely reduced lateral root production and leaf cell expansion (Weijers *et al.*, 2005; Wilmoth *et al.*, 2005), though the root hair phenotype of these mutants has not been reported. It will be interesting to ascertain whether ARF7 and ARF19 have aberrant root hair elongation, and are downstream targets of SHY2/IAA3 and/or phyB regulated root hair elongation, or whether SHY2 regulates root hair length by repression of other ARFs.

I have proposed that phyB and *SHY2* interact to regulate microtubule stability, and that *AXR1* mediated SHY2 turnover may be an important process in this mechanism. This work therefore provides insights into the integration of phytochrome and auxin signals, and how these important regulators of plant development are able to moderate cellular elongation through regulation of microtubule dynamics. Colocalisation analysis of *SHY2:*SHY2:YFP and phyB:PHYB:GFP is ongoing, and will be important in understanding whether the interaction of phyB and SHY2 occurs *in vivo*, and is not merely an artefact of *in vitro* pulldown assay technique. As regulation of Aux/IAA ARF optimal pairs is at least partially

regulated by transcriptional control of the distribution of each protein, this work will indicate whether similar genetic interactions occur in the shoot as well as the root. Study of root hair cells therefore has broader implications for understanding the role of both auxin and phytochrome in plant development.

Chapter 5

Discussion



Nuclear speckles of light grown phyB:phyB:GFP seedlings

CHAPTER 5

DISCUSSION

Phytochrome signalling has been studied extensively in the shoot, and we are beginning to understand the complex nature of the pathways involved in shoot physiology. In contrast, little attention has been paid to light control of root development, largely due to the assumption that these underground structures would have little requirement for photoreceptor action. However, co-ordinated development requires shoot-root communication, providing the possibility that phytochrome action in the shoot may influence root development. Recently, Kiss *et al.* (2003b) demonstrated that phytochromes control root phototropism, providing evidence for a role for phytochromes acting from within the root. Thus, phytochromes may impact upon root development via action in both the shoot and the root. I set out to assess the impact of phytochrome signalling on root development and to establish the nature of the signalling pathways involved.

Shoot Phytochromes Act Collectively To Regulate Lateral Root Production

Given the extensive evidence linking phytochrome and auxin signalling pathways (reviewed in chapter 1), and the published roles for auxin in lateral root emergence (Bhalerao *et al.*, 2002), I was interested to know if the phytochromes had effected lateral root production. I took a genetic approach and grew seedlings lacking single or multiple phytochrome species, and assessed the lateral root production of these mutants over a five day time course.

My results revealed complex interactions between the phytochrome species underlying this response. *phyA*, *phyB* and *phyE* mutant seedlings produced fewer lateral roots than the wild type at any given time point, with *phyB* mutants having the most pronounced deficiency (30% fewer lateral roots than the wild type during the experimental timeframe). Thus, phyB has a major role, and phyA and phyE lesser roles as positive regulators of lateral root emergence. *phyD* seedlings produced more lateral roots than the wild type during the experimental period, suggesting that phyD is a negative regulator of this response, acting antagonistically to phyA, phyB and phyE to control lateral root emergence. Double mutant analysis revealed that *phyB* was epistatic to *phyD,* and *phyE* epistatic to *phyB* for this response. Coordinated action of the phytochromes also occurs in the shoot in response to light (Casal *et al.*, 2003). For example, under red light, phyB is the major regulator of hypocotyl elongation and cotyledon expansion, with other phytochromes acting largely, but not only, redundantly alongside phyB to control these responses (Qin *et al.*, 1997; Franklin *et al.*, 2003; Monte *et al.*, 2003; Franklin and Whitelam, 2004).

Under my growth conditions, in line with previous reports, *hy5* seedlings produce more lateral roots than wild type plants (Oyama *et al.*, 1997; Cluis *et al.*, 2004). Thus, in marked contrast to their interactions in hypocotyl elongation, phyB and HY5 act antagonistically to regulate lateral root emergence (Koornneef *et al.*, 1980; Ang and Deng, 1994). Unlike the phytochrome mutants, the rate of lateral root production in *hy5* sharply decreases after day 9, with *hy5* seedlings producing fewer lateral roots that wild type at day 11. Furthermore, *hy5* seedlings produce fewer lateral roots than wild type when grown in the dark. My data therefore indicate that either the phytochromes act independently of HY5 to moderate lateral root growth, or suggest a highly complex relationship between these genes for this response. Thus phytochrome regulation of lateral root emergence is likely to utilise different signalling pathways that those already identified for shoot development.

Phytochromes act over long distances, via modification of auxin flux, to regulate lateral root production

Auxin transport is heavily implicated in the development of lateral roots. Auxin is synthesised in the shoot of young seedlings, and is transported to the root through the vasculature, and via polar auxin transport, where it stimulates the production of lateral roots (Bhalerao *et al.*, 2002; Marchant *et al.*, 2002; Ljung *et al.*, 2005). I was interested to know if phytochromes regulated lateral root emergence by manipulating of this auxin pulse. I examined seedlings expressing the *DR5::GUS* construct under low R:FR ratio light, to deplete levels of active phytochrome. Under these conditions, both Col *DR5::GUS* and Ler seedlings had elongated hypocotyls and reduced lateral root production when compared to high R:FR conditions. Interestingly, lateral root development was not drastically different between these treatments, possibly reflecting the complex interactions between the phytochromes in the control of lateral root emergence. This result may however indicate that the Pfr/Ptot threshold required to generate a marked root effect was not been achieved. Under these conditions I did observe notable changes in the distribution of *DR5::GUS* expression. Thus, physiological changes in the seedling in response to low R:FR ratio light are accompanied by a redistribution of auxin, which accumulates in the shoot and is lost from the root. These data therefore support my hypothesis that phytochrome moderates lateral root development by regulating the flux of auxin from shoot to root. My data are in line with a model proposed by Morelli and Ruberti (1996) in which low R:FR ratio light triggers a lateral redistribution of auxin within the shoot, consequently leading to reduced auxin reaching the root. This model was based on studies on *ATHB-2*, a homeobox gene upregulated in response to EOD-FR treatment, under the control of phyB and phyE (Carabelli *et al.*, 1993; Franklin *et al.*, 2003). Overexpression of *ATHB-2* results in reduced lateral root production, a phenotype which may be rescued by topical IAA application,

suggesting that the *ATHB2* OX phenotype may result from an auxin deficiency (Steindler *et al.*, 1999). ATHB2 is therefore a downstream component of the model, as *ATHB2* levels correlate with phenotype severity (Steindler *et al.*, 1999).

Phytochromes are expressed in the root and respond to light

The phytochromes are expressed in the root, though at lower levels than in the shoot (Hall *et al.*, 2001; Toth *et al.*, 2001), and the expression patterns of *PHYB, PHYD* and *PHYE::GUS* changes during seedling development (Somers and Quail, 1995; Goosey *et al.*, 1997). I wanted to know the pattern of phytochrome expression in my growth conditions, and whether this pattern changed over my experimental timeframe. *PHYA, PHYD, and PHYE::LUC* exhibited similar patterns of expression at both 7 and 10 days, present throughout the root with strong foci of expression at the tips of the primary and lateral roots. Low levels of expression could be seen in the roots of both *PHYB* and *PHYC::LUC.* This may reflect low levels of expression of these genes, but may also be characteristic of these particular transgenic lines, particularly given the prominent roles identified for phyB in the regulation of root-specific responses (Reed *et al.*, 1993; Kiss *et al.*, 2003b). Analysis of expression pattern using qRT-PCR may help resolve this issue.

Phytochrome protein-GFP fusions have been used to analyse the cellular distribution of phytochrome. Activation by light induces a conformational change to the active Pfr form and movement to the nucleus where diffuse staining and/or bright speckles of staining are observed (Sakamoto and Nagatani, 1996; Kircher *et al.*, 1999; Yamaguchi *et al.*, 1999; Gil *et al.*, 2000; Kim *et al.*, 2000; Chen *et al.*, 2003). This behaviour is seen in each of the phytochrome species (Kircher *et al.*, 2002). Several studies have shown that the formation of subnuclear foci is linked to phytochrome function, with mutant forms of phyA and phyB exhibiting reduced or no physiological activity do not aggregate to nuclear foci (Kircher *et*

al., 2002; Chen *et al.*, 2003). Indeed, the size of nuclear foci (speckles) correlates with the magnitude of the physiological response (Chen *et al.*, 2003). However, speckle formation is not linked to all physiological responses. Recent work by Matsushita *et al.*, (2003) showed that a truncated phyB (with only the N-terminal) which translocates to the nucleus but does not aggregate to nuclear foci, is more physiologically active than full length phyB. Furthermore, under low fluence rate red light conditions, which generate diffuse phyB nuclear staining, plants are still light responsive (Chen *et al.*, 2003). As these studies have been conducted in shoot cells I wanted to know whether phytochromes behaved similarly in root cells. I have shown this is indeed the case. In response to light, PHYA-E::GFP moved from the cytosol to the nucleus, and formed subnuclear speckles in root epidermal cells. Furthermore, I observed diffuse nuclear PHYB::GFP in root epidermal cells of seedlings grown under low fluence rate red light. These data indicate that phytochrome within the root may be physiologically active, and could have a role in the regulation of local responses. Recent reports suggest that this is indeed the case. Kiss *et al.*, (2003a; 2003b) used selective shading experiments to show that red light mediated root phototropism was regulated by phytochromes acting from within the root. Furthermore, Correll and Kiss (2005) showed that inhibition of primary root elongation is mediated by phytochromes acting from the shoot and from within the root.

Phytochromes A, D and E act redundantly with phyB to regulate root hair elongation

I have shown that the phytochromes act together to regulate lateral root emergence, acting over long distances through the modification of a shoot derived auxin pulse. Given that phytochromes are present, and light regulated in roots, I was interested to know if phytochromes regulated other aspects of root development. Reed *et al.*, (1993) identified a role for phyB as a negative regulator of root hair elongation. Likewise, the light signalling component HY5 has also been shown to negatively regulate root hair elongation (Oyama *et*

al., 1997, 2002). I was interested to know if phyB acted alone in this response, or whether the other phytochrome species contributed. To this end, I compared the root hair phenotypes of mutants lacking individual or multiple phytochrome species to wild type seedlings. Consistent with previous reports, under my experimental conditions *phyB* and *hy5* had long root hair phenotypes (Reed *et al.*, 1993; Oyama *et al.*, 1997, 2002). Interestingly, *phyA*, *phyD* and *phyE* mutants were essentially wild type for this response. These phytochromes are important in the regulation of root hair length, as double mutant analysis revealed that *phyA* and *phyE* were both epistatic to *phyB*. phyA and phyE are therefore implicated in the control of root hair elongation by phyB.

Phytochrome regulates lateral root and root hair growth by different mechanisms

The genetic relationships underlying lateral root emergence and root hair elongation are different, suggesting that these two phytochrome-controlled responses are regulated by different mechanisms. Furthermore, auxin has been shown to promote root hair elongation, yet my analysis of seedlings expressing *DR5::GUS* showed that low R:FR ratio light reduces root auxin levels, yet enhances root hair elongation. Thus phytochromes cannot act solely by moderating the shoot-root auxin pulse to regulate root development. This provides the possibility that phytochrome is controlling root development via an additional shoot-derived signal, or that phytochrome is operating within the root system itself to control aspects of development. Local expression of phytochrome in the root hair cells of a *phyB* mutant, either using a root hair specific promoter or by a biolistic approach will indicate whether or not this phytochrome is able to act locally to regulate this response.

Phytochromes May Be Able To Act In The Pr Form To Regulate Root Growth

I was interested to know whether phytochrome regulation of lateral root emergence and root hair development required light inputs and photoconversion to the active Pfr form, or

whether these phenotypes were examples of light independent, Pr phytochrome functions. I therefore analysed lateral root development and root hair elongation in seedlings overexpressing a mutated phyB that was unable to incorporate the chromophore (C7g), and compared these phenotypes to seedlings over expressing wild type phyB (ABO), and wild type and *phyB* seedlings (Hennig *et al.*, 2001). C7g seedlings produced similar numbers of lateral roots to ABO seedlings, indicating that the chromophore, and light sensing capabilities of phyB were not required for this phenotype. Furthermore, C7g seedlings produced shorter root hairs than wild type, the opposite phenotype to *phyB* mutants, indicating that Pr phytochrome was also able to regulate root hair length. However, whilst these results indicate that Pr phytochrome can be active in roots, they must be interpreted cautiously, as the C7g is an overexpresser line and consequently not only contains modified C7g, but also native and intact phyB. The observed responses could be a result of dominant negative effects whereby competition between modified and native phyB blocks signalling through developmental pathways. However, C7g seedlings were compared to the ABO line for all analyses, indicating that for these responses C7g phyB is able to function as a phyB overexpresser line.

Differences in lateral root emergence between phytochrome null mutants were also maintained when grown in the dark, providing further support for light independent phytochrome functions. Interestingly, Correll and Kiss (2005) have recently shown that root elongation is mediated by phyA and phyB, and that phenotypic differences between these two mutants are maintained when grown in the dark. These data therefore provide further evidence of a light independent role for phytochrome in root development.

PhyB and SHY2 Interact to Regulate Root Hair Elongation

I was curious to understand the mechanism by which phyB regulated root hair elongation. Like *phyB*, *shy2-2* gain of function mutants have longer root hairs than wild type. phyB has been shown to interact *in vitro* with SHY2 (Tian *et al.*, 2003), and the *shy2-2* mutation was originally isolated as a suppresser of the *phyB* and *hy2* phenotypes (Reed *et al.*, 1998). Thus, *SHY2* seemed was a candidate in phyB mediated root hair elongation. I analysed the root hairs of *shy2-2phyB-1* double mutants to assess if these two genes acted together. If these two genes acted independently to control this phenotypes, then root hairs of the double mutant would be expected to be longer than either parental single mutant. The *shy2-2phyB-1* root hairs were in fact deformed, leading me to surmise that these two genes did in fact work together to regulate root hair elongation.

The root hairs of *shy2-2phyB-1* seedlings are branched and wavy, but do not occur ectopically, and are even in diameter along most of their length. These root hairs bear a striking resemblance to those of seedlings treated with the microtubule disrupting drugs oryzalin and taxol, and to *mor1* and *mrh2* mutants, which are disrupted in microtubule organisation (Whittington *et al.*, 2001; Jones *et al.*, 2006). I therefore propose that the genetic interaction between *SHY2* and *PHYB* is important for regulation of microtubule dynamics.

Interestingly, SHY2 stability is increased in *axr1* mutants. AXR1 is a nuclear localised protein with homology to an E1 ubiquitin ligase and is required for the activation of RUB1/NEDD8 (Leyser *et al.*, 1993), and consequently for the targeted degradation of proteins. Targeted neddylation of SHY2, and therefore AXR1 mediated regulation of SHY2 turnover, may be an important process in the regulation root hair elongation. PhyB may interact with AXR1 to regulate SHY2 stability, or may act downstream alongside SHY2 to

regulate root hair elongation. It will be interesting to determine whether SHY2 stability is altered in *phyB* mutants. Furthermore, as IAA proteins act by regulating the activity of specific ARF transcription factors, it will be interesting to identify ARFs that may be misexpressed in *shy2-2* and *shy2-2phyB-1* mutants. Isolation of ARFs downstream of both SHY2 and phyB could identify a direct link between phyB and the cytoskeleton, not only in root hairs but also in other cells. To this end, I am preparing root tissue of *shy2-2*, *phyB-1*, *shy2-2phyB-1* and Ler (wild type) seedlings for microarray analysis which will help resolve these questions.

Novel Perspectives On Phytochrome Signalling

I have shown that phytochromes have wide ranging effects on root development (summarised in Figure 5.1). My studies of phytochrome localisation have shown that phytochromes are expressed in roots, and that they respond to light in a similar manner to shoot phytochromes. Coordinated action of all the phytochrome species enables fine control over developmental processes in the root, as it does in the shoot. My work highlights the distinction between shoot and root, and indicates that whilst many aspects of phytochrome signalling are conserved between shoot and root, phytochromes also utilise previously uncharacterised signalling pathways to regulate root development.

I have shown that phytochromes are able to act over very long distances, with shoot phytochromes moderating lateral root emergence by regulation of a shoot derived auxin pulse. Whilst phytochromes have been shown to signal between cells, and between organs of the shoot (Tanaka *et al.*, 2002), signalling over such long distances has not previously been shown for the phytochromes. It will be interesting to understand the mechanism of underlying this long distance signalling. There are several lines of evidence linking

phytochrome and auxin signalling, though the basis of phytochrome regulation of auxin flux is poorly understood. Several genes known to be involved in auxin transport are regulated by phytochromes, including members of the PIN family of auxin efflux carriers, and AUX1-like influx proteins. Thus, phytochrome may regulate lateral root production by changing the distribution, quantity or sensitivity of components of the auxin transport system, and consequently by changing the transport of auxin throughout the plant. Recent studies have implicated the MDR/PGP subclass of ABC transporters in auxin transport. Members of this gene family regulate the distribution of PIN proteins (Noh *et al.*, 2003), and catalyse the efflux of auxin in yeast and mammalian cell systems (Geisler *et al.*, 2005). MDR-like proteins are implicated in light signalling, with *br2* mutants of maize (a *pgp1* homologue) exhibiting light specific regulation of auxin transport (Multani *et al.*, 2003), and *pgp1* mutants exhibiting a red light specific short hypocotyl phenotype (Sidler *et al.*, 1998). One member of this family, AtPGP4 has also recently been shown to be involved in lateral root and root hair development (Santelia *et al.*, 2005). Phytochromes may control lateral root development by regulating auxin flux by direct moderation of PIN proteins, or indirectly through regulation of MDR family genes. Analysis of the expression of these genes in phytochrome null mutants, and under different R:FR ratio lights by qPCR and through analysis of reporter gene fusions will help to unravel this mechanism.

I have also provided evidence that phytochromes are able to act in the Pr form to regulate root development. Such a suggestion is controversial, and challenges the central dogma that phytochrome acts solely as a light receptor, and in the Pfr form. Whilst such a mode of action has been alluded to in the past, reports have not been followed up by the wider community (Liscum and Hangarter, 1993; Kim *et al.*, 1998). However, bacterial phytochromes do act in the Pr form (Karniol and Vierstra, 2003). Given that bacterial and plant phytochromes are derived from a common ancestor, it is tempting to speculate that

plant phytochromes retain some Pr functions. My hypothesis is therefore in direct contention with widely held beliefs of phytochrome function. I have obtained transgenic lines expressing chromophore deficient phyB, expressed in a *phyB* null background.

Analysis of the root phenotypes of these lines will allow me to understand whether the chromophore is required for a subset of root responses, without the problems potentially caused by dominant negative effects in the C7g lines (Hennig *et al.*, 2001).

Through my analysis of phytochrome regulation of root hair length has led to the identification of a link between phytochrome and auxin signalling pathways and the cytoskeleton. Both phytochrome and auxin are important regulators of elongation growth, and the analysis of root hair development provides an ideal system for understanding how these two signals are able to control development.

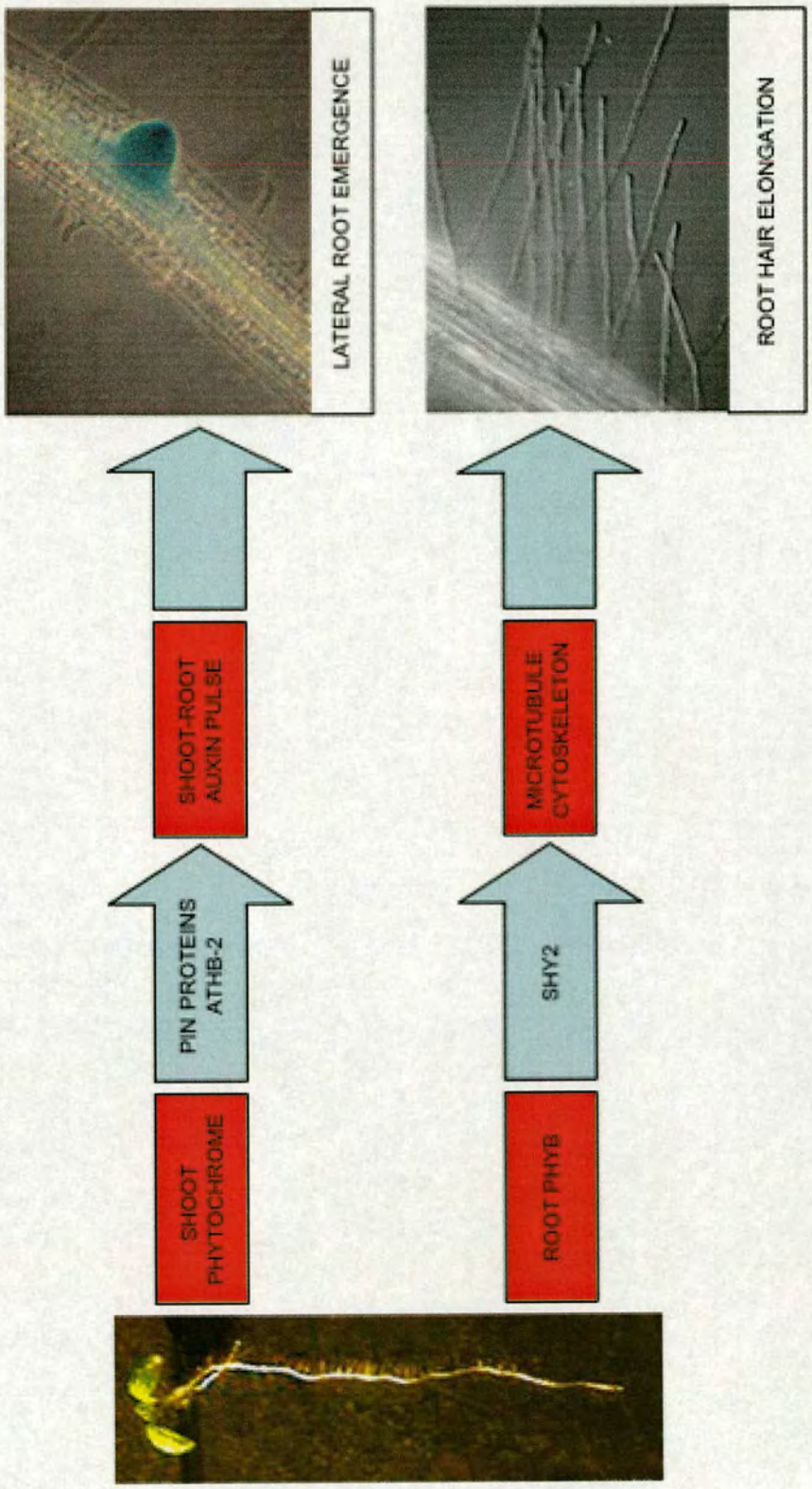


Figure 5.1: Summary of the role of phytochromes in root development
Phytochromes are able to moderate lateral root emergence, at least partially through the modification of a shoot derived auxin pulse Phytochromes may moderate auxin flux by stimulating the redistribution of auxin to tissues of the shoot (potentially acting through the homeobox gene *ATHB-2*), or by directly regulating the auxin transport machinery (for example, by regulation of the PIN proteins). Phytochromes are also regulators of root hair elongation. Double mutant analysis reveals that phyB interacts with SHY2/IAA3 to moderate this response, probably by regulating the stability of microtubule cytoskeleton.

Environmental Significance

It is perhaps intuitive that shoot-root communication is essential to synchronise plant growth and development. I have shown that light, a potent regulator of shoot development, has a role in this process, acting through the phytochrome photoreceptors. My data, along with that of others suggests that phytochrome action is not just confined to the shoot, but also acts within the root system to control cell elongation. It is currently unclear when these signalling pathways would be used in the natural habitat. One possibility is that Arabidopsis roots are exposed to light more frequently than we imagine. Arabidopsis is a pioneer species in the wild, and often grows over compacted substrates or in shallow soils. Under such conditions roots are easily exposed by wind or rain, thus, photoreceptor action may be important to alter growth in response to exposure. Alternatively, the phytochromes may in fact regulate development in roots beneath the soil. There is evidence that roots are not in fact in darkness, but are actually exposed to a unique light environment. Axial conduction of light through vascular root tissues has been observed in several species (Mandoli and Briggs, 1982, 1984; Sun *et al.*, 2005). Vascular tissues, and to a lesser extent cells of the cortex and pith, of herbaceous stems are able to conduct light (Sun *et al.*, 2005). Furthermore, wavelengths in the far-red and infra-red are the most efficiently transported. Chlorophyll in the shoot absorbs mainly shorter wavelengths in the electromagnetic spectrum, which may also enrich the internal light environment for these longer wavelengths. Thus, when growing in soil in the wild, Arabidopsis roots are likely to be exposed to far-red light conducted from the shoot to the root, via the tissues of the stem. In this situation the phytochromes are ideally placed to act as light sensors in the root.

Summary

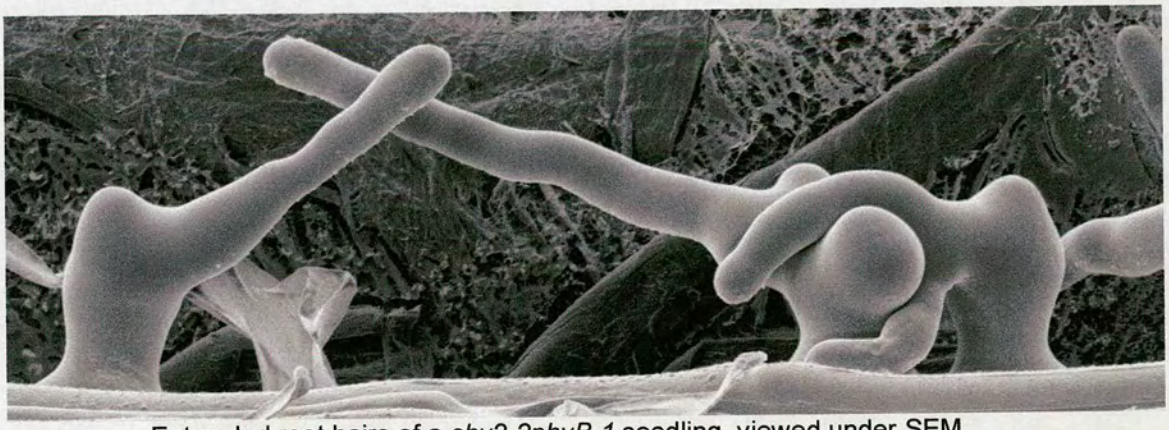
Over the course of my research, I have taken a novel approach to understanding phytochrome signalling. I have identified long distance signalling roles for shoot

phytochrome, enabling synchronisation of lateral root emergence with the light environment of the shoot. Furthermore, I have shown that phytochromes are present and light regulated in roots, and propose that these root phytochromes are able act locally to regulate root hair elongation. My analysis of the interaction between *phyB* and *shy2-2* implicates these genes in the regulation of microtubule stability, and consequently of cytoskeleton organisation. I have also provided evidence that the Pr form of phytochrome, previously thought to be physiologically inactive, does in fact have important roles in the regulation of root development.

My work into phytochrome regulation of root development is changing our perceptions of the roles of phytochromes in plants. I have identified new developmental processes regulated by phytochrome a have opened many new lines of enquiry into an exciting new area of photobiology.

Chapter 6

Materials and Methods



Entangled root hairs of a *shy2-2phyB-1* seedling, viewed under SEM

CHAPTER 6

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All studies were carried out in the *Arabidopsis thaliana* Landsberg *erecta* (Ler) or Columbia (Col) accessions. The phytochrome mutant alleles used in this study were (Ler) *phyA-201* (Nagatani et al., 1993), *phyB-1* (Reed et al., 1993), *phyD-1* (Aukerman et al., 1997) and *phyE-1* (Devlin et al., 1998). Double mutants were created by genetic crossing as described in (Devlin *et al.*, 1998; 1999). We used *PHYA-E promoter::LUC* lines and the lines expressing 35S:PHYA-E:GFP translational fusions. These were a gift from Professor F. Nagy (Hungarian Academy of Sciences, Szeged, Hugary) and have been previously described (Toth et al., 2001; Kircher et al., 2002). *PHYB*:PHYB:GFP lines were a kind gift from Professor A. Nagatani (Kyoto University, Kyoto, Japan). The *DR5::GUS* line, containing a synthetic auxin responsive promoter fused to the *GUS* reporter gene, in the Col genetic background as has previously described (Ulmasov et al., 1997).

For all experiments, seeds were surface sterilized in 20% (v/v) bleach for 5 minutes. After 3 washes in distilled water, seeds were sown on plates containing Hoaglands No.2 basal salts medium pH5.7 [SigmaAldrich, Gillingham, UK], 1% (w/v) sucrose, and 0.5% (w/v) phytagel [SigmaAldrich, Gillingham, UK]. Seeds were then stratified in complete darkness for 3 days at 4°C before transfer to specific growth conditions. Plates were positioned vertically to allow root growth along the gel surface. For plant growth, we used a plant growth cabinet [Snijders Scientific, Tilburg, Netherlands] in all experiments under 16 hours

of white light (fluence rate of 100 μmol m^{-2} s^{-1}) and a temperature of 18°C ±0.5°C. High/low R:FR ratio light experiments were performed in climate controlled growth rooms, also 18°C ±0.5°C, and at a photon fluence rate of 70 μmol m^{-2} $s^{-1,}$ and 16 hour photoperiod. Supplementary FR light was supplied by light emitting diode (LED) arrays to create a low R:FR ratio of 0.126. Light quantity, and quality were measured with a StellarNet EPP2000 spectroradiometer [Astranet systems, Cambridge, UK].

Physiological Analysis

Root hairs and lateral roots were viewed with a stereomicroscope [MZFL*III,* Leica Microsystems, Wetzlar, Germany]. For root hair measurements, images from the mature zone were recorded with a digital camera [2.2.1.,Diagnostic Instruments Inc., Michigan, USA]. Root hair length was measured with ImageJ [NIH, NCBI]. The number of emerged lateral roots was counted daily between 7 and 11 days after transfer to white light.

Reporter Gene Analysis

To visualise *PHYA-E::promoter::LUC* expression seedlings were sprayed with 5mM luciferin 5 minutes before analysis of LUC expression pattern. Seedlings were imaged using an intensified CCD camera [Hamamatsu VIM, Hamamatsu City, Japan]. Images were processed using NIH image [RSB, NIMH, USA], and Photoshop 8 [Adobe Systems Inc, San Jose, CA].

To assess the cellular properties of PHYA-E we analysed the effect of light on the cellular location and characteristics of 35S::PHYA-E::GFP, and *PHYB:*PHYB:GFP. Seedlings were

viewed using an Eclipse confocal microscope [Nikon Corporation, Japan]. Colour was artificially added using Photoshop 8 [Adobe Systems, San Jose CA].

For histochemical analysis of GUS activity, Arabidopsis seedlings were incubated overnight at 37°C in GUS reaction buffer (0.5mM 5-bromo-4-chloro-3-indolyl-β-D-glucoronic acid in 100mM sodium phosphate pH7.0). Stained seedlings were cleared with 70% ethanol overnight before being mounted in glycerol and viewed under a DMLB stereomicroscope [Leica Microsystems, Wetzlar, Germany]. Representative seedlings were photographed using a digital camera [Coolpix 4200, Nikon, Tokyo, Japan].

GUS staining was quantified through analysis of MUG (4-Methylumbelliferyl glucoronide) fluorescence. Seedlings were severed at the shoot root junction, and shoot and root tissues were then incubated separately in GUS extraction buffer (1mM MUG [SigmaAldrich, Gillingham, UK], 50mM sodium phosphate, pH7, 10mM EDTA, 0.1% SDS, 0.1% Triton X-100) at 37°C for 6 hours, before the reaction was stopped (1M sodium carbonate). MUG fluorescence of each sample was measured on Fluorolite1000 fluorometer [Dynatech Laboratories, Chantilly, VA].

Fluoroscein Diacetate Staining

Cytoplasm of living root hairs was visualised by staining seedlings in a 0.005% (w/v) solution of fluorescein diacetate [SigmaAldrich, Gillingham, UK] according to Galway *et al.*, (1997). Stained seedlings were mounted in glycerol and viewed using a DMLB stereomicroscope [Leica Microsystems, Wetzlar, Germany].

Auxin Biosynthesis Assay

IAA levels and biosynthesis were quantified according to Ljung *et al.*, (2005). Seedlings were grown on vertical plates as described previously. After 7 days seedlings were removed from plates and incubated for a further 24hours in liquid culture medium containing 30% deuterium oxide (Hoaglands salts, 1%sucrose, in 30%D20). 2mm sections from 50 root tips were then dissected and immediately frozen in 0.05M $NaPO_4$, pH 7.0. Tissues was immediately frozen in N_2, and sent to Karin Ljung (Umea) for quantification of IAA levels and IAA biosynthesis. For this analysis, tissue was homogenized using a MixerMill [Retsch GmbH, Haan, Germany] in buffer (0.05mM $NaPO_4$ pH7.0, 0.02% DEDTCA, 125pg $^{13}C_6$-IAA). Samples were then applied to a preconditioned Varian Bond Elut-C18 (50mg.ml-1) column. The column was washed with 10%MeOH in 1%HA, and the liquid phase removed by vacuum. Samples were eluted in MeOH and dried by speed vacuum. Dried samples were dissolved in isopropanol and MeCl, and trimethylsilyl-diazomethane (in hexane) was added. Samples were then left for 30 minutes at room temperature. Excess diazomethane was removed by addition of HAc in hexane, and samples were dried to completion in a speed-vac. Samples were transferred to GC-vials and acetonitirile was added and evaporated. Samples were then mixed with 15µl acetonitrile and 15µl BSTFA (1%TMCS) and incubated at 70°C for 30 minutes before evaporation. Finally samples were dissolved in heptane and analyzed by GC-MS.

MOLECULAR BIOLOGY TECHNIQUES

Extraction of DNA

Genomic DNA was extracted from plant tissue following the Edwards method (Edwards *et al.*, 1991). Tissue was macerated in extraction buffer (200mM Tris HCL, 250mM NaCl, 25mM EDTA, 0.5%SDS) and centrifuged. The supernatant was mixed with isopropanol for 5 minutes before further centrifugation. The resulting pellet was washed in 70% and 100% ethanol before drying, and was resuspended in distilled water.

Plasmid Purification

Plasmid DNA was extracted from *E.coli* using QIAprep Minipreps [Qiagen, Crawley, UK] following manufacturers instructions. This kit employs the modified alkaline lysis method of Birnboim and Doly (1979).

Polymerase Chain Reaction (PCR)

PCR reaction mixes were set up in sterile tubes. Each reaction consisted of 2µl template DNA, 2µl dNTPs (10mM), 2.5µl $MgCl_2$ and 1.25µl each of the forward and reverse primers (100pmolμl^{-1}) [MWG-Biotech UK Ltd, Milton Keynes]. 0.2µl redTaq DNA polymerase [Bioline, London, UK] was added and the mixture transferred to a thermal cycler [PTC 100, MJ Research, Waltham, MA]. Amplification conditions (table 6.1) were varied to suit the size of the expected product. The extension times were calculated as 60s/1Kb product. Reaction annealing temperatures were the lower annealing temperature of a primer pair. PCR products were checked by electrophoresis.

Agarose Gel Electrophoresis

1% agarose (w/v) was dissolved in 1x TAE (0.04 M Tris-acetate, 0.001M EDTA) buffer by heating. The agarose was allowed to cool before the addition of 0.5% w/v ethidium bromide (10mg/ml). The mixture was then poured into an agarose gel-forming tray containing a 'comb' well former. Once the agarose gel had set and the 'comb' removed, the gel was place into a horizontal electrophoresis chamber and TBE (Tris-borate-EDTA) was added until the level covered the gel.

DNA loading buffer was added to the samples to be analysed and a DNA marker was also prepared. For gel electrophoresis, samples were loaded into the wells formed in the agarose gel and run at 100 V for 90 minutes. DNA was visualised using UV light and the images recorded photographically.

DNA Gel Extraction

Removal of DNA from agarose gels was achieved using the QIAquick gel extraction kit [Qiagen, Crawley, UK].

TA Cloning

Gel extraction and PCR products were ligated into pCR®2.1-TOPO® [Invitrogen, Paisley, UK] by TA cloning. DNA was mixed with salt solution (1.2M NaCl, 0.06M $MgCl_2$) and pCR2.1-TOPO vector (10ng) and incubated for 5 minutes at room temperature. The reaction mixture was then transformed into competent cells.

Step	Cycle Name	Temperature	Time	Number of Cycles
1	Initial Denaturation	94°C	5 mins	1
2	Denaturation Annealing Extension	94°C various 72°C	1 minute 1 minute various	35
3	Final Extension	72°C	20 mins	1

Table 6.1: Outline of PCR cycles used for isolation of DNA sequences for SHY2 and YFP

Primer Name	Orientation	Sequence	Incorporated Restriction Site	Function/Target
shy2for	Forward	CGTCCCGGGGCTAGCTGCTATAATCAACCAGCG	XmaI, NheI	Amplication of genomic SHY2
shy2rev	Reverse	CTTGCGGCCGCTACACCACAGCCTAAACC	NotI	Amplification of genomic SHY2
FPfor	Forward	CTTGCGGCCGCTATGGTGAGCAAGGGCGAGGAGCTGT	NotI	Amplification of YFP
FPrev.	Reverse	GCGCCGCGGGGTCACCTTTACTTGTACAGC	SmaI	Amplification of YFP

Table 6.2: Primers used in the construction of pGreen:SHY2:YFP

Competent Cells

One Shot® TOP 10 Chemically Competent *E. coli* [Invitrogen, Paisley, UK] are commercially available competent *E. coli* for cloning. *E. coli* were incubated aerobically on Luria-Bertani (LB) medium agar plates or in LB broth with shaking at 200 r.p.m. Antibiotics were added as appropriate.

Transformation of *E.coli*

Frozen competent cells were taken from –70°C and thawed until just defrosted on ice. 5-10μg of DNA was added to the 100μl of cells, and the mixture incubated on ice for 30 minutes. Cells were heat shocked at 42°C for 60 seconds before adding 250μl of SOC medium (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose). Cells were then grown at 37°C for at 2 hours. 70μl of the transformation reaction was then spread onto LB agar plates containing the appropriate antibiotics and were left overnight at 37°C

Digestions

Restriction enzymes were from New England Biolabs [Beverly, MA]. Double digests were performed by incubating DNA with 0.25μl of each restriction enzyme in 1.5μl of enzyme buffer, in the presence of 1%BSA. Reactions were incubated at 37°C for 2 hours.

Ligation

T4 DNA Ligase [Promega, Madison, WI] was used for annealing fragments into plasmid vectors. A 1:3 molar ratio of vector to insert was used when cloning. Insert and vector DNA were combined in a 10μl reaction containing 2.5μl of 10 x T4 DNA Ligase Buffer and 1μl T4 DNA Ligase. The reaction was placed in a thermal cycler [PTC 100, MJ Research, Waltham, MA] for 200 cycles, alternating between 10°C and 30°C for 30 seconds each.

Agrobacterium Mediated Plant Transformation

Agrobacterium tumifaciens (strain GV 3130-C58) was transformed using the freeze-thaw method. Plasmid was extracted from *E.coli* using minipreps, and mixed with *Agrobacterium*, and frozen in N_2 before incubation at 37°C for 5 minutes. Precultures were then made by adding 1ml LB broth and incubated for 4 hours at 30°C. Cells were then grown for 3 days on selective LB agarose medium containing rifampicin ($100\mu g.ml^{-1}$), gentamycin ($10\mu g.ml^{-1}$) and kanamycin ($50\mu g.ml^{-1}$). Cultures of *Agrobacterium* containing the plasmid were grown overnight with identical concentrations of selective agents. This culture was grown to 500ml at 28°C, and was spun down and resuspended in 5% sucrose, 0.02% silwet L-77. Flowers of wild type (Ler), Bpro (*PHYB*:PHYB:GFP) and *shy2-31* were dipped in this solution and covered for 48 hours. Mature seed were harvested and dried, ready for antibiotic selection with kanamycin.

Chapter 7

References

Root hairs views under differential interference contrast

CHAPTER 7

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